Intracellular localization of an active green fluorescent protein-tagged Pho84 phosphate permease in *Saccharomyces cerevisiae*

Jens Petersson[a,b], Johanna Pattison[a,b], Arthur L. Kruckeberg[c], Jan A. Berden[c], Bengt L. Persson[a,b,*]

[a] *Department of Engineering and Natural Sciences, Växjö University, 351 95 Växjö, Sweden*
[b] *Department of Biochemistry, Stockholm University, 106 91 Stockholm, Sweden*
[c] *E.C. Slater Institute/BioCentrum Amsterdam, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands*

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Abstract Green fluorescent protein (GFP) from *Aequorea victoria* was used as an in vivo reporter protein when fused to the carboxy-terminus of the Pho84 phosphate permease of *Saccharomyces cerevisiae*. Both components of the fusion protein displayed their native functions and revealed a cellular localization and degradation of the Pho84-GFP chimera consistent with the behavior of the wild-type Pho84 protein. The GFP-tagged chimera allowed for a detection of conditions under which the Pho84 transporter is localized to its functional environment, i.e. the plasma membrane, and conditions linked to relocation of the protein to the vacuole for degradation. By use of the methodology described, GFP should be useful in studies of localization and degradation also of other membrane proteins in vivo.

Key words: Phosphate transport; Green fluorescent protein; Yeast

1. Introduction

The Pho84 phosphate permease of *Saccharomyces cerevisiae*, encoded by the *PHO84* [1], belongs to a family of phosphate:H^+ symporters (PHS) and is a member of the major facilitator superfamily [2]. This hydrophobic integral membrane protein consisting of 587 amino acid residues catalyzes the coupled transport (symport) of phosphate and H^+ across the yeast plasma membrane by conversion of the energy stored in an electrochemical H^+ gradient into energy for translocation of phosphate into the cell. Based on hydropathy analysis, a secondary structure model has been proposed in which the transporter contains 12 transmembrane segments connected by hydrophilic loops with the N- and C-termini on the cytoplasmic face (see [3,4] for recent reviews). Expression of the transporter in *S. cerevisiae* cells is subject to control by the *PHO* regulatory pathway (reviewed in [5]). When the cells are starved for phosphate, derepression is mediated through the *PHO* system, leading to initiation of transcription of *PHO84* and synthesis of the Pho84 permease [1,6]. This derepressible high-affinity phosphate permease proposed to have a K_m for external phosphate in the low μM range [7] thus adds to the activity of a low-affinity (K_m approximately 1 mM) PHS proposed to be constitutively expressed [8]. In contrast to these H^+-coupled phosphate transporters being maximally active at a pH close to 4.5, the Pho89 permease, a derepressible high-affinity (K_m 0.5 μM) Na^+-coupled phosphate permease with a pH optimum of 9.5, has been identified [9]. Activation of the high-affinity transport systems thus allows the cells to scavenge phosphate from the environment under conditions of phosphate limitation. Recently, evidence has been presented that additional gene products act together with the Pho84 permease in the phosphate acquisition process (see [5]). These proteins, i.e. Pho86 [10], Pho87 [11], Pho88 [12] and Gtr1, a putative GTP-binding protein [13], have been proposed to be associated with the phosphate transport system, possibly serving as a receptor for phosphate signals from the environment [5] or otherwise act as a regulatory unit in the adaptation to changing metabolic conditions in the cell. Previous studies of intact yeast cells [1,6] and inverted plasma membrane vesicles [14] have verified that the Pho84 permease is localized in the plasma membrane and takes part in the catalysis of a bidirectional H^+-coupled phosphate uptake. However, unequivocal evidence that the Pho84 protein contains the phosphate translocation pathway has been obtained by *Escherichia coli* overexpression of a histidine-tagged Pho84 protein which, after purification and reconstitution into proteoliposomes, was shown to catalyze an uncoupler-sensitive accumulation of phosphate [15]. Although previous studies have indicated that the expression and degradation of the Pho84 permease and its activity is regulated by the level of extracellular phosphate [6], the details of this regulation are so far poorly understood. In the present study, we have investigated the role of extracellular phosphate in its regulation of the cellular localization and activation of the permease. For this purpose, a Pho84 permease fusion has been constructed with the green fluorescent protein (GFP) from *Aequorea victoria* (see [16] for a recent review on GFP) attached to the C-terminus (Fig. 1). By this means, expression and localization of the active Pho84-GFP chimera have been monitored by fluorescence microscopy. The use of this technology has allowed for the first analysis of Pho84 dynamics in live cells. In the present study, we show, to our knowledge, for the first time that a GFP fusion to the H^+-coupled Pho84 phosphate permease of *S. cerevisiae* can be stably expressed as an active plasma membrane transport protein and that its subcellular localization is regulated by the level of extracellular phosphate.

*Corresponding author. Fax: (46) (470) 70 87 56.
E-mail: bengt.persson@itn.vxu.se

Abbreviations: CMAC-Arg, 7-amino-4-chloromethylcoumaryl-L-arginine amide dihydrochloride; GFP, green fluorescent protein

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2. Materials and methods

2.1. Strains and DNA constructs

The *S. cerevisiae* wild-type (WT) strain used was CEN.PK113-7D (*MAT*a *MAL*2-8^c *SUC*2). To localize the Pho84 permease in viable cells, we introduced the DNA sequence encoding the GFP in-frame immediately downstream of the CEN.PK113-7D chromosomal *PHO84* open reading frame (ORF) using a polymerase chain reaction (PCR) targeting technique [17,18], resulting in a strain, designated JP11, expressing Pho84 fused with GFP(S65T) at its C-termini. Sense oligonucleotide primer (5′-AATGACATTGAATCTTCCAGCCC-ATCTCAACTTCAACATGAAGCAAGTAAAGGA-GAAGAACT-TTTC-3′, 66 nucleotides) and antisense oligonucleotide primer (5′-GTATTATTTGTTCTAGTTTACAAGTTTTAGTGCATCTTTGA-GGCTTGGATGGCGGCGTTAGT-ATC-3′m, 65 nucleotides) were used to PCR amplify a GFP-kanMX6 cassette DNA sequence using the pFA6-GFP(S65T)-kanMX6 plasmid as template. The primers were designed also to be homologous with the last 45 nucleotides of the *PHO84* ORF and the first 46 nucleotides downstream of the *PHO84* ORF, respectively. The PCR product of the correct size (2.6 kbp) was detected by agarose gel electrophoresis and transformed by the lithium acetate procedure as described [19] and integrated in the yeast genome by homologous recombination. After phenotypic expression in YP-maltose medium for 1 h at 30°C, transformants were selected on YP-maltose plates containing G418 at a concentration of 200 mg/l. Positive colonies were identified after 4 days of incubation at 30°C and were re-streaked onto YPD-G418 plates for further selection. Verification of positive colonies was accomplished by qualitative PCR and Western blot analysis.

2.2. Expression of Pho84-GFP chimera

S. cerevisiae cells expressing WT Pho84 or Pho84-GFP chimera (JP11) were pre-cultivated aerobically for 12 h in YPD at 30°C, washed twice with water and inoculated in high phosphate (HP_i) or low phosphate (LP_i) media [20]. Cells were grown aerobically at 30°C and samples for microscopy analyses and phosphate assays were withdrawn at indicated time points.

2.3. Microscopy analyses

Samples from *S. cerevisiae* cells expressing WT Pho84 or Pho84-GFP chimera (JP11) grown in LP_i medium (WT and JP11) and HP_i medium (JP11) were mixed with an equal volume of melted agarose (1%), immobilized on a pre-warmed slide and cooled to 4°C prior to analysis. The U-M41012 filter (Olympus) was used for the excitation of GFP(S65T). For staining of vacuolar lumen, 7-amino-4-chloromethylcoumaryl-L-arginine amide dihydrochloride (CMAC-Arg) (Molecular Probes) was used according to the manufacturer's protocol and visualized with an Omega optical bandpass XF03 filter (Molecular Probes). The cells were monitored using a 100/1.25 Oil Ph3 objective on an Olympus BX40 microscope (100 W Hg source) equipped with a BX-FLA filter wheel and a cooled CCD camera (DP10). For image capture, Olympus DP-Soft 3.0 software was used. The final images were produced using Adobe Photoshop and Micrografx PhotoMagic.

2.4. Determination of phosphate transport

Phosphate uptake in intact *S. cerevisiae* cells expressing WT Pho84 or Pho84-GFP chimera (JP11) in LP_i medium was assayed by the addition of 1 μl [^{32}P]orthophosphate (carrier-free, 0.18 Ci/μmol, 1 mCi = 37 MBq) to 30 μl aliquots of cells, suspended to 3 mg of cells (wet weight)/30 μl of a buffer containing 25 mM Tris-succinate, pH 4.5, and 3% glucose, to a final concentration of 0.22 mM P_i essentially as described [6]. In the kinetic analyses of the Pho84 WT protein and the Pho84-GFP chimera, P_i uptake in cells harvested at an A_{600} of 3.5 was assayed at phosphate concentrations ranging from 0.01 to 1.0 mM P_i.

2.5. Determination of extracellular phosphate

The extracellular phosphate concentration was determined spectrophotometrically at 735 nm essentially as described [21].

2.6. Electrophoresis and Western blot analysis

WT and JP11 cells grown to an A_{600} of 3.5 in LP_i medium as described in Section 2.2 were collected by centrifugation at 5500×*g*, 4°C for 10 min. Cells were resuspended in 20 mM Tris-HCl (pH 8.0), containing 0.2% Triton X-100 and 0.5% sodium dodecyl sulfate (SDS), followed by four sonication pulses of 30 s each by use of a micro-tip-type sonicator (VirSonic model 60, The Virtis Company) at 80% efficiency. The lysates were centrifuged for 10 min at 5500×*g* and 4°C and the supernatants were passed through a 0.2 μm filter. The protein concentrations were determined by use of the Bio-Rad *DC* Protein Assay kit (Bio-Rad) and bovine serum albumin as standard, adjusted to 0.25 mg/ml and mixed with sample buffer prior to separation by SDS-polyacrylamide gel electrophoresis (PAGE) using a 12% Laemmli system [22]. Immunoblotting was carried out on poly-(vinylidene difluoride) membranes (Immobilon-P, Millipore) according to the Western blotting protocol (Amersham Pharmacia Biotech). Immunological detection of the Pho84-GFP chimeric protein was accomplished by use of anti-*A. victoria* GFP polyclonal rabbit antibody (Molecular Probes) and anti-rabbit Ig donkey antibody-conjugated horseradish peroxidase (ECL, Amersham Pharmacia Biotech). After a short incubation with chemiluminescence substrates, the blot was exposed to film for 1 min.

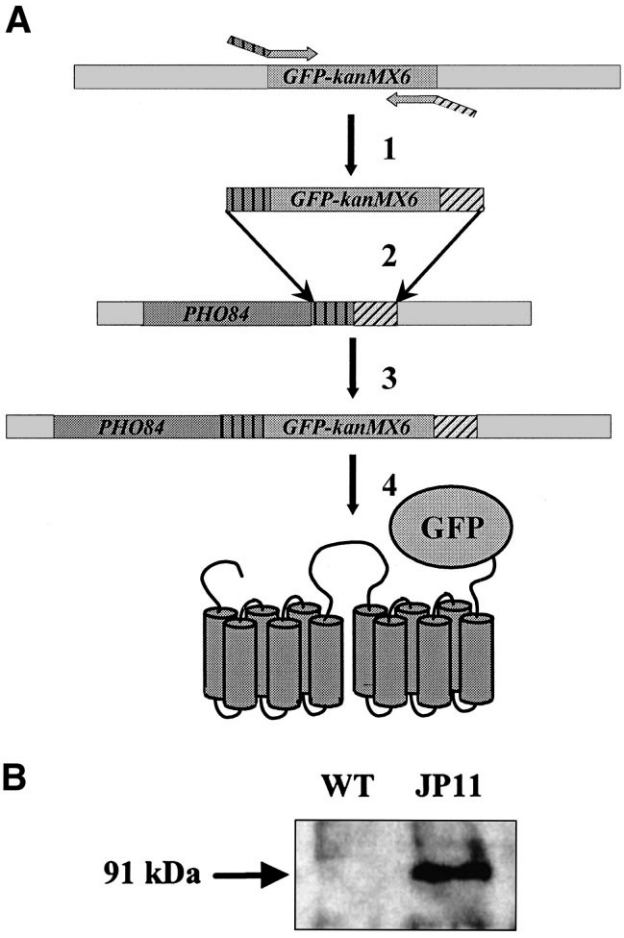


Fig. 1. Schematic representation of construction of Pho84-GFP fusion permease. (A) Oligonucleotide primers designed not only to complement the ends of the DNA cassette to be amplified (*GFP-kanMX6*) but also to show homologies with the last nucleotides of the *PHO84* ORF and the first nucleotides immediately downstream of the *PHO84* ORF (shown with horizontal and tilted dashed lines, respectively) were used in a PCR cycle yielding *GFP-kanMX6* DNA fragments (1). The PCR products were transformed into the WT CEN.PK113-7D strain (2) and integrated in the yeast genome by homologous recombination (3), resulting in the strain JP11 harboring the chimeric gene expressing the Pho84-GFP fusion protein (4) under the same regulatory control as WT Pho84 protein. (B) Immunological detection of the Pho84-GFP fusion protein in JP11 cells, using anti-GFP primary antibodies, shows the presence of a protein band at the expected molecular size of 91 kDa, which is absent in WT cells.

3. Results

3.1. Construction and expression of a Pho84-GFP fusion protein

In Fig. 1, the strategy used for the creation of a *PHO84-GFP* chimeric gene is outlined. The C-terminal fusion of GFP to the Pho84 phosphate permease was accomplished by use of a *GFP-kanMX6* cassette containing the coding sequences for GFP and antibiotic resistance. By PCR amplification, the flanking regions of the *GFP-kanMX6* sequence [17,18] were modified to be homologous to the 45 last nucleotides of the *PHO84* gene except for the stop codon (TAA). The other flanking end of the cassette was homologous to the 46 nucleotides of the genomic DNA sequence located immediately downstream of the *PHO84* gene. After transformation of the amplified cassette into *S. cerevisiae* CEN.PK113-7D WT cells, a homologous in-frame recombinant DNA construct was formed between the *PHO84* and *GFP* genes. The presence of the *PHO84-GFP* construct in the new strain, JP11, was verified by qualitative PCR analysis of the fused gene construct (not shown) and immunodetection of the GFP moiety of a 91 kDa band separated by SDS-PAGE. Immunodetection of this protein band in WT cells by use of the same GFP antibody was absent, strongly indicating that the antibodies specifically recognized a GFP-modified protein at the expected size of the Pho84-GFP chimera (Fig. 1B).

3.2. Functional analysis of the Pho84-GFP protein

In order to verify that the Pho84-GFP fusion protein was functionally expressed in LP_i medium and targeted to the plasma membrane of the yeast cells, the P_i transport activity of the fusion protein was compared with that of the Pho84 protein in WT cells (Fig. 2). In both cell types, P_i uptake was at its maximum in cells grown to an A_{600} of 3, after which the transport activity declined. Although the transport activity catalyzed by cells harboring the fusion protein was about 3-fold lower than that observed in WT cells, the Pho84-GFP mutant is clearly competent of P_i transport. Both strains exhibited an identical growth pattern up to an A_{600} of 2, after which the growth of the JP11 strain lagged behind that of the WT strain (not shown). It is likely that the differences observed at higher A_{600} values are due to the presence of the modified Pho84 protein.

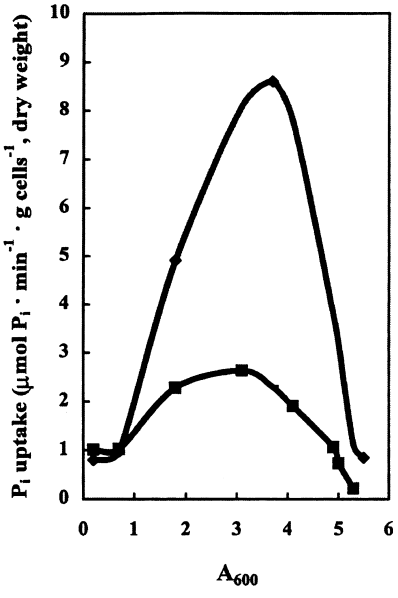
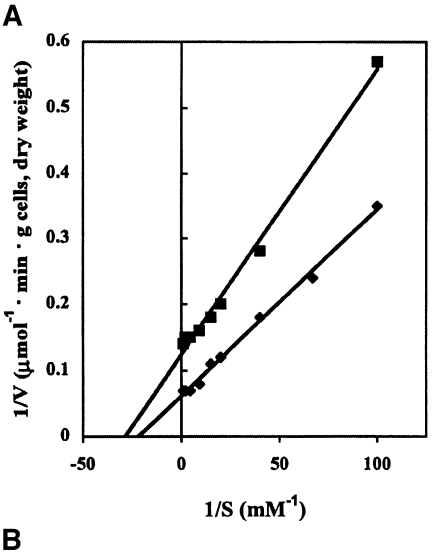


Fig. 2. Phosphate uptake by WT and mutant JP11 cells. WT (♦) and mutant (■) cells grown in LP_i medium were assayed for P_i transport activity at different A_{600} values as described in Section 2. Transport activity was determined as μmol P_i taken up per g (dry weight) of cells.



B

	K_m	V_{max}
WT	45 μM	16 μmol · min^{-1} · g $cells^{-1}$
JP11	34 μM	8 μmol · min^{-1} · g $cells^{-1}$

Fig. 3. Determination of K_m and V_{max} for the WT Pho84 (♦) and the Pho84-GFP chimera (■). (A) A Lineweaver-Burk plot was used to estimate the K_m and V_{max} of the Pho84 transporter. $1/V$ is the reciprocal of the maximum rate of P_i uptake and $1/S$ is the reciprocal of the P_i concentration analyzed. Transport activity was determined as μmol P_i taken up per g (dry weight) of cells. (B) K_m and V_{max} for the WT Pho84 and the Pho84-GFP chimera.

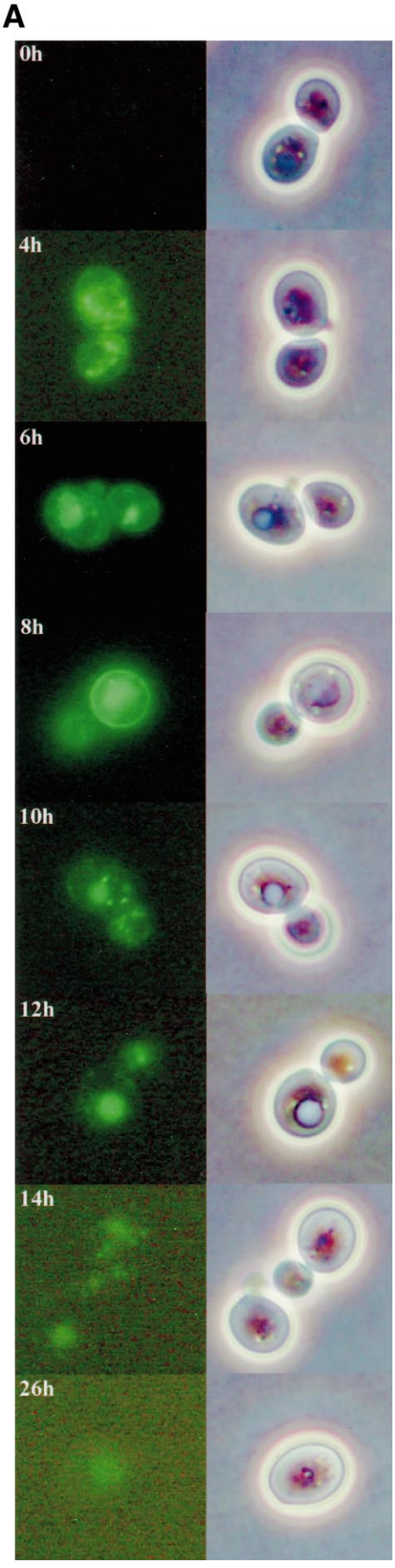
3.3. Comparison of the kinetics of the Pho84 proteins

To further scrutinize the kinetical parameters affecting the altered transport rate in the JP11 strain at A_{600} of 3.5, a Lineweaver-Burk analysis of the Pho84 and the Pho84-GFP transporters was performed (Fig. 3). It can be seen that both K_m and V_{max} of the Pho84-GFP protein were affected as compared to the Pho84 protein. As can be seen in Fig. 3B, V_{max} of the transporter in the mutant JP11 strain was reduced 2-fold while the K_m was reduced from 45 μM for the WT strain to 34 μM for the JP11 strain.

3.4. Subcellular localization of the Pho84-GFP protein

Expression and subcellular localization of the Pho84-GFP protein of the JP11 strain was monitored by fluorescence microscopy. JP11 cells grown in LP_i and HP_i medium were collected at various time points during growth and mixed with pre-melted low-percentage agarose for immobilization of the sample specimen for microscopy analysis. Representative photographs of Pho84-GFP fluorescence and corresponding phase contrast of JP11 cells are shown in Fig. 4A. As can be seen, expression of the Pho84-GFP protein upon growth in LP_i medium, initially containing approximately 300 μM P_i, resulted in a strong intracellular fluorescence where the fluorescent signal probably associated with endoplasmic reticu-

lum-localized de novo-synthesized protein appeared during the early exponential growth phase (4 h). At prolonged growth, the intensity of the GFP signal in the plasma membrane increased and reached a maximal intensity at 8 h. At 10 h of growth, or longer, the total intensity diminished and was found to be transiently associated with an intracellular compartment after which it was fully depleted (14 and 26 h). Phase contrast analyses of the Pho84-GFP expressing cells was performed in order to show the boundaries of the cell and its organellar structure. It is noteworthy that the cellular compartment to which the GFP signal is localized upon prolonged growth (10 h) increases in size during exponential growth. Growth of JP11 cells in HP_i medium containing at least 15 mM of P_i did not, as expected, yield a GFP signal (not shown). Fig. 4B illustrates the growth of JP11 cells in LP_i medium as used for the microscopy analyses. It can be seen that the cells have a log-phase growth rate of 0.6 absorbance units per hour and reach an A_{600} equivalent to 5 after 13 h of growth. During the initial lag-phase, an accumulation of the extracellular 300 μM P_i contained in the LP_i medium was initiated followed by an accelerated P_i accumulation during the early exponential growth phase. After 6 h of growth, the external P_i supply was exhausted. When the external P_i was reduced to a concentration of 80 μM or below, the P_i transport catalyzed by the cells at pH 4.5 was activated from an initial rate of 1 μmol/min/g cells to a maximum transport rate of 2.7 μmol/min/g cells at 8 h of growth. After 8 h, equivalent to an A_{600} of 3.2, the P_i transport activity rapidly declined. When the cells had reached an A_{600} of 4.5, the P_i transport has been inhibited by 50% and at prolonged growth (26 h) by 90%. In order to verify the subcellular localization of the Pho84-GFP protein during endocytosis, the vacuolar lumen marker, CMAC-Arg, was used for fluorescent staining of the cells harvested throughout the growth curve (Fig. 4B). Shown in Fig. 4C are photographs of CMAC-Arg staining of cells harvested at an A_{600} of 3 and A_{600} of 5. The fact that the vacuolar staining coincided with the subcellular organelle seen by phase contrast microscopy and by GFP fluorescence during inactivation of the Pho84 phosphate permease chimera confirm the identity of the GFP-targeted organelle. These results clearly indicate an active vacuolar degradation pathway of the Pho84 permease during inactivation of the transporter as has been observed with several other plasma membrane transporters [23,24]. Interestingly, as judged by GFP fluorescence microscopy (Fig. 4A), a significant portion of the Pho84-GFP chimera expressed at 6 h of growth, i.e. under conditions when GFP localization of an active permease to the plasma membrane is favored, seems to be sorted also to the vacuole.



4. Discussion

GFP modification of a number of proteins has proven to be highly informative with respect to their intracellular regulation and distribution. This fluorescence-based technique has thus allowed for in vivo analyses which otherwise have been difficult to perform. The reporter function of GFP is useful in the study of localization and turnover of membrane proteins (see [16] for a review). By construction of a C-terminal fusion of GFP to the Pho84 phosphate transporter of the *S. cerevisiae* plasma membrane, we have been able to follow the expression of the phosphate transporter and its localization to

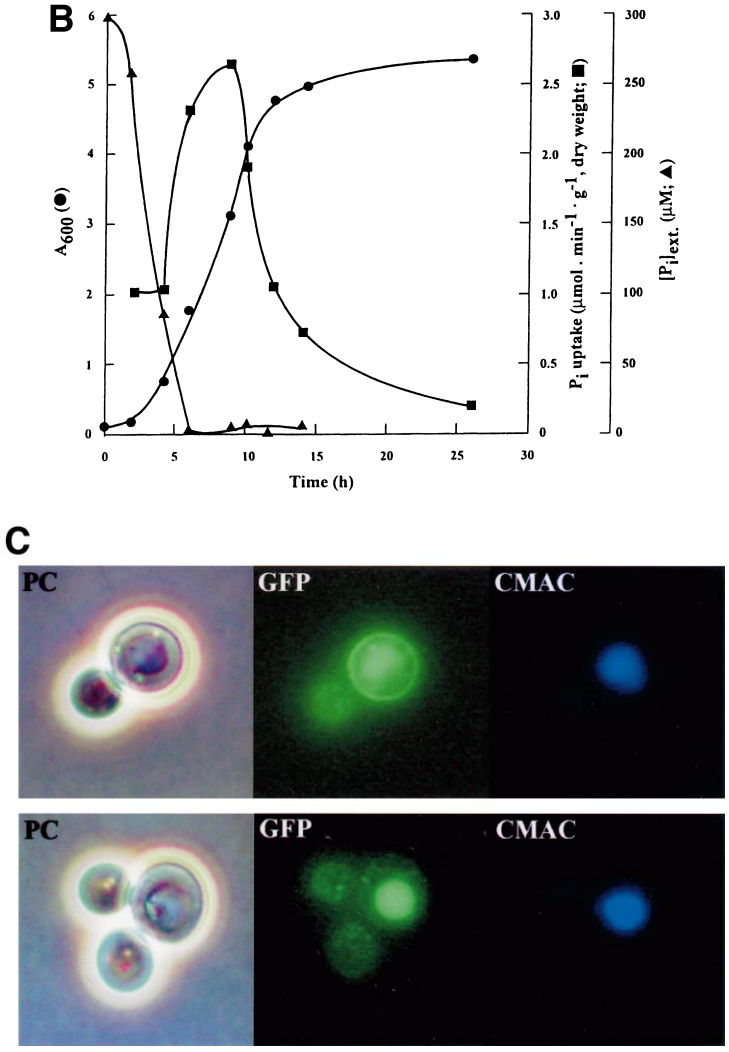


Fig. 4. Localization and activity of the Pho84-GFP chimera during P_i starvation. (A) Photographs of a time-course study showing the localization of fluorescent Pho84-GFP and (B) phosphate uptake by JP11 cells during growth (●) in LP_i medium. At specified time intervals, samples were withdrawn and assayed for inorganic phosphate uptake (■) and the supernatants were used for external phosphate determination (▲). (C) Study of the relocation pathway, in which CMAC-Arg was used to stain the vacuolar lumen of JP11 cells grown to an A_{600} of 3 and 5, clearly shows vacuolar accumulation of Pho84-GFP fusion permease paralleled by inactivation of the transporter.

the plasma membrane during derepression of the *PHO84-GFP* gene. Previous observations have shown that derepression of the *PHO84* gene occurs when the cell meets limitations in P_i supply [1] and that the synthesis and activation of the permease occurs when the external P_i concentration is lower than 100 μM [6]. Studies of several different plasma membrane nutrient transporters suggest that inactivation and/or degradation of such permeases relies on a general mechanism by which the cell responds to changes in the availability of the nutrients. Degradation of the maltose [25–28], galactose [29], uracil [30], general amino acid [31] and Hxt2 [32], Hxt6 and Hxt7 [33] hexose permeases has been proposed to occur via endocytosis followed by transport to the vacuole where the protein is degraded. In a previous study of a WT *S. cerevisiae* strain, we have shown that de novo synthesis of the Pho84 permease is halted when external P_i has been exhausted and that the protein localized in the plasma membrane is subjected to rapid degradation [6]. Results obtained by use of the Pho84-GFP fusion construct described in this study show that the Pho84 phosphate permease fusion protein is synthesized and routed to the plasma membrane when the cells meet derepressive concentrations of external phosphate. Furthermore, the localization of the permease is shown to be controlled by the prevalent concentration of external P_i and that depletion of the P_i supply results in disappearance of the protein from the plasma membrane and relocalization to the vacuole where the protein undergoes degradation. Several lines of evidence support this conclusion. Clearly, transcription of the *PHO84-GFP* gene is controlled by the *PHO* pathway as in the case of the WT-expressed *PHO84* gene. Stable translation of the *PHO84-GFP* transcript occurs when the JP11 cells are grown in a LP_i medium as judged by the GFP immunoreactivity detected by Western blot analysis. In contrast, JP11 cells grown in HP_i medium do not express the Pho84-GFP protein. Moreover, the expressed fusion protein clearly maintains the activities of both its components, i.e. the fusion protein catalyzes a high-affinity H^+-coupled P_i symport with the same pH optimum as the Pho84 protein, and the

GFP domain maintains its native fluorescent characteristics. The Pho84-GFP protein was shown to be targeted and inserted into the plasma membrane in a functional state. Although the fusion protein catalyzes cellular P_i accumulation with a 3-fold lower rate than that observed for the WT protein, the external P_i control of the derepressive synthesis and activation of the fusion protein is identical to that of the Pho84 protein. Therefore, the reason for the lower transport rate of the fusion protein may be accounted for by two possible scenarios. Either the activity of the fusion protein localized to the plasma membrane is hampered by the introduced GFP domain or, alternatively, a portion of the synthesized fusion protein is maintained in the cytosolic compartment and is not localized to the plasma membrane. It is, in this context, noteworthy that GFP fluorescence can be observed not only in the plasma membrane at the time point of maximum transport activity, corresponding to an A_{600} of 3, but also in intracellular compartments, suggesting the presence of a population of the Pho84-GFP protein not localized in the plasma membrane. Although the latter interpretation is supported by the observed 2-fold decrease in the V_{max} of the transport activity observed in the JP11 strain as compared to the WT strain, an influence of the GFP domain on the catalytic properties of the enzyme cannot be excluded. It deserves to be pointed out that it is at the present time unknown whether also the Pho84 protein in WT cells shows a cellular partitioning between the cytosolic compartments and the plasma membrane. It is, however, clear that synthesis and localization of the Pho84-GFP protein to the plasma membrane are paralleled by P_i starvation-induced activation of the Pho84-mediated P_i uptake. It is evident that the fusion protein is not expressed under conditions when the activity of the constitutively expressed low-affinity P_i transport system is adequate for cellular P_i acquisition. As can be seen, cellular starvation of nutrients such as P_i during growth results in an enlargement of the vacuole, an observation in agreement with a common morphology when cells are energy-depleted and/or starved for nutrients [34,35]. Inactivation of the Pho84-mediated P_i transport triggered by exhaustion of external P_i, previously observed also in the case of Pho84 expressed in WT cells [6], is shown to be paralleled by a decrease of fluorescent Pho84-GFP in the plasma membrane and an accumulation of the fluorescent signal in the vacuole. The disappearance of the fluorescent signal in the vacuole when cells have reached a stationary growth phase suggests that this organelle is active in proteolytic breakdown of the Pho84 protein. This study thus adds the Pho84 phosphate permease to the list of plasma membrane proteins that undergo sorting to the vacuole for degradation. Whether this process involves phosphorylation of specific serine residues followed by ubiquitination still remains to be established.

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