Mutagenic and functional analysis of the C-terminus of *Saccharomyces cerevisiae* Pho84 phosphate transporter

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Abstract **A widely accepted mechanism for selective degradation of plasma membrane proteins is via ubiquitination and/or phosphorylation events. Such a regulated degradation has previously been suggested to rely on the presence of a specific SINNDAKSS sequence within the protein. Modification of a partly conserved SINNDAKSS-like sequence in the C-terminal tail of the Pho84 phosphate transporter, in combination with C-terminal fusion of green fluorescent protein or a MYC epitope, were used to evaluate the presence of this sequence and its role in the regulated degradation. The functional Pho84 mutants in which this SINNDAKSS-like sequence was altered or truncated were subjected to degradation like that of the wild type, suggesting that degradation of the Pho84 protein is regulated by factors other than properties of this sequence.** © **2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Phosphate transporter; Pho84; *PHO* gene; Plasma membrane

1. Introduction

The uptake of inorganic phosphate (P_i) in *Saccharomyces cerevisiae* relies on the activity of at least three different permeases (reviewed in [1]). Of these, two are derepressible high-affinity transporters encoded by the *PHO84* [2] and *PHO89* [3] genes, and one is a kinetically identified low-affinity transporter believed to be constitutively expressed (see [4]). As P_i uptake at the cell membrane is a crucial step in P_i acquisition, the *PHO84* and the *PHO89* genes encoding the high-affinity transporters are tightly regulated at transcriptional level by the *PHO* regulatory pathway [5–8] in response to external P_i levels [6,9] in order to ensure sufficient P_i for cellular needs. Of the two, the life cycle of the Pho84 protein, active predominantly at acidic conditions, has been more thoroughly described. At external P_i concentrations lower than 100 μM the *PHO84* gene is derepressed, resulting in synthesis and targeting of the protein to the plasma membrane. After prolonged growth, when external P_i or glucose is exhausted, the P_i uptake activity declines as a consequence of drastically lowered levels of mRNA and the protein itself [6]. Construction of a chimeric Pho84-green fluorescent protein ($Pho84^{WT}$-GFP) and its expression from the chromosomal location of the *PHO84* under control of its native upstream promoter have allowed for detailed intracellular localization studies of the trafficking of Pho84 protein during this process [10]. Moreover, these studies indicated the existence of a down-regulatory pathway with the vacuole being the terminal destination for degradation of the Pho84 protein under these restrictive P_i conditions, as has been shown to be the case for several other plasma membrane receptors and nutrient transporters destined for degradation in this acidic compartment [11–16]. Endocytosis and the degradation process have been shown to be initiated for some of these proteins by covalent modification with ubiquitin and/or phosphorylation (reviewed in [17,18]. One of the most extensively studied *S. cerevisiae* proteins in this regard is the α-factor pheromone receptor, the Ste2 protein. The amino acid sequence SINNDAKSS, located within the C-terminal tail of this plasma membrane protein, constitutes the target site for signaling endocytosis whereby the lysine residue contained within this sequence is ubiquitinated, an event preceded by phosphorylation of the serine residues ([12] and [19], respectively). A similar sequence (nkNNDieSS; capital letters indicate conserved residues) is partly conserved in the C-terminal tail of the polytopic membrane-spanning Pho84 protein. The similarity of the two protein domains urged us to analyze the in vivo role of this sequence in localization and function of the Pho84 protein. To investigate the role of these conserved residues we used an experimental approach involving substitution and deletion mutagenesis of the nkNNDieSS C-terminal sequence and homologous recombination of wild-type and mutant chimeras into the yeast genome.

2. Materials and methods

2.1. Materials and strains

[^{32}P]orthophosphate (carrier-free), horseradish peroxidase (HRP)-conjugated anti-mouse-Ig-antibody (from sheep) and enhanced chemiluminescence detection kit were obtained from Amersham-Pharmacia Biotech, Sweden. Anti-myc antibodies were obtained from Invitrogen, The Netherlands. TaqPlusLong polymerase was from Stratagene. Zymolyase 100T was obtained from Sekagaku America. All mutagenic primers were purchased from TAG Copenhagen, Denmark. Haploid, prototrophic *S. cerevisiae* CEN.PK113-7D (*MAT*a *MAL2-8c SUC2*) and its auxotrophic derivative CEN.PK113-5D (*MAT*a *MAL2-8c SUC2 ura3-52*) were kindly provided by P. Kötter, Frankfurt, Germany. The JP11 cells in which the DNA sequence encoding the

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GFP was introduced in-frame immediately downstream of the CEN.PK113-7D chromosomal *PHO84* open reading frame, resulting in a strain expressing Pho84 fused with GFP at its C-termini, have previously been described [10].

2.2. Epitope tagging

The *PHO84* gene was tagged with a stretch of DNA encoding six consecutive histidine residues and a tandem MYC epitope using polymerase chain reaction (PCR) technology essentially as described in [20]. The integration cassette was PCR amplified from pU6H2MYC with oligonucleotide primers #1 and #3 (Table 1) and subsequently transformed into CEN.PK113-5D cells wherein homologous recombination occurred. After selection on YPD-geneticin (200 μg/ml) plates, colonies were re-streaked on fresh YPD-geneticin plates and positives were verified using qualitative PCR and Western blot analyses.

2.3. Construction of carboxy-terminally truncated/mutated Pho84 derivatives

Derivatives of genomically expressed Pho84 were constructed in combination with MYC epitope tagging in CEN.PK113-5D cells as described above or with a C-terminal addition of GFP in CEN.PK113-7D cells as described previously [10]. For the generation of truncated Pho84 (Δ570–587) constructs, primers #2 and #3 (Pho84^{-18aa}-MYC) and #4 and #9 (Pho84^{-18aa}-GFP) were used (Table 1). Mutations of C-terminal Pho84 amino acids, combined with GFP addition, were introduced via PCR mutageneses using sense primers #5 (Pho84^{KR}-GFP containing the replacement K571R), #6 (Pho84^{4SA}-GFP containing the replacements S577A, S578A, S579A and S581A), #7 (Pho84$^{KR,\ 2SA}$-GFP containing the replacements K571R, S577A and S578A) and #8 (Pho84$^{KR,\ 4SA}$-GFP containing the replacements K571R, S577A, S578A, S579A and S581A) and with #9 as antisense primer (Table 1).

2.4. Growth and expression

S. cerevisiae cells expressing Pho84^{WT}, Pho84^{WT}-MYC or Pho84^{-18aa}-MYC were pre-cultivated aerobically for 12 h in YPD medium at 30°C, washed twice with ice-cold distilled water, and inoculated in low-phosphate (LP_i) media [21]. Cells expressing Pho84^{WT}-GFP, Pho84^{-18aa}-GFP, Pho84^{KR}-GFP, Pho84^{4SA}-GFP, Pho84$^{KR,\ 2SA}$-GFP or Pho84$^{KR,\ 4SA}$-GFP were inoculated in LP_i defined media, containing initially 250 μM P_i [22]. Cells were grown aerobically at 30°C and samples for P_i incorporation, phosphate determination, Western blot and fluorescence microscopy analyses were withdrawn at indicated time points.

2.5. Determination of phosphate transport and extracellular phosphate

Phosphate uptake in intact *S. cerevisiae* cells expressing Pho84^{WT}, Pho84^{WT}-MYC or Pho84^{-18aa}-MYC grown in LP_i medium and determination of extracellular phosphate were assayed as described previously [23].

2.6. Microscopy analyses

Samples from *S. cerevisiae* cells expressing the Pho84-GFP chimera and mutants thereof grown in LP_i defined medium were analyzed by fluorescence microscopy essentially as described in [10].

2.7. Electrophoresis and Western blot analysis

Cells grown to specified A_{600} values in LP_i medium were collected by centrifugation at 5500×*g*, 4°C for 10 min, resuspended and gently agitated in pre-spheroplasting buffer (0.5 M sorbitol, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 2.5 mM dithiothreitol, and 2.5 mM phenyl methyl sulfonylfluoride for 15 min at 25°C. Following centrifugation, the cells were incubated in 10 ml of spheroplasting buffer (pre-spheroplasting buffer modified to contain 1 M sorbitol and 1 mg/5 A_{600} units of Zymolyase) for 1.5 h. The spheroplasts were pelleted and resuspended in extraction buffer composed of 9 M urea, 2.5% (v/v) Triton-X100 and 0.5% CHAPS. Samples were adjusted to 2.5 mg/ml and mixed with sample buffer prior to separation by SDS–PAGE using a 10% Laemmli system [24]. Immunoblotting was carried out on poly(vinylidene difluoride) membranes (Immobilon-P, Millipore) according to the Western blotting protocol (Amersham Pharmacia Biotech.). Use of anti-GFP monoclonal mouse antibody (Roche Molecular Biochemicals) and HRP-conjugated anti-mouse-Ig-antibody allowed for immunological detection of the Pho84^{WT}-GFP chimeric protein. Immunological detection of the Pho84^{WT}-MYC construct was carried out with anti-myc antibodies and treated as described above for the GFP detection.

2.8. Protein determination

Protein was assayed by use of the commercially available D_c Protein Assay kit (Bio-Rad). Bovine serum albumin was used as standard.

3. Results and discussion

3.1. Internalization and degradation of the Pho84 protein

Using the previously constructed Pho84^{WT}-GFP transporter contained in JP11 cells [10] we have analyzed the degradative internalization of the protein by fluorescence microscopy. Synthesis and sorting of the chimera to the cell periphery was clearly visualized in JP11 cells grown to maximal expression in LP_i defined medium (Fig. 1A). Addition of repressive amounts of P_i to the cells resulted in a rapid internalization of the protein. After 20 min the membrane has been essentially depleted of transporter molecules and after 80 min all molecules have been routed to the intracellular compartment previously identified as the vacuole [10]. To investigate whether the observed depletion of the Pho84^{WT}-GFP protein fluorescent signal of the cell periphery was accompanied by internalization of the full-length chimera, we treated JP11 cells expressing the Pho84^{WT}-GFP protein with P_i for various time periods followed by electrophoretic separation of solubilized proteins and immunoblot analyses using anti-GFP monoclonal mouse antibody (Fig. 1B). As shown in Fig. 1B, addition of repressible amounts (10 mM) of P_i resulted in a rapid time-dependent disappearance of the full-length chimera migrating with a molecular mass of 91 kDa without any clear accumulation of proteolytic products during the first 30 min of treatment. The peptide bands of approximately 55–60 kDa observed are possibly proteolytic cleavage products of the chimeric protein which after 30 min are further degraded to the peptide doublet seen at approximately 38–40 kDa. The intense signal detected at approximately 30 kDa corresponds to the GFP domain of the chimera. As indicated by our fluo-

Table 1
Oligonucleotides designed for creation of truncated and site-directed amino acid mutations of GFP-tagged, and full-length and truncated MYC-tagged Pho84 constructs

No.	Sequence (5′→3′)
1	AATGACATTGAATCTTCCAGCCCATCTCAACTTCAACATGAAGCAtcccaccaccatcatcac
2	GAGCTATACCACGATGAAATCGATCCTGCTACGCTAAACTTCAGAtcccaccaccatcatcatcac
3	GTATTATTTGTTCTAGTTTACAAGTTTTAGTGCATCTTTGAGGCTTactatagggagaccggcagatc
4	GAGCTATACCACGATGAAATCGATCCTGCTACGCTAAACTTCAGAagtaaaggagaagaacttttc
5	A**GA**AATAATGACATTGAATCTTCCAGCCCATCTCAACTTCAACATGAAGCAagtaaaggagaagaacttttc
6	AATGACATTGAA**G**CT**G**CC**GC**CCCA**G**CTCAACTTCAACATGAAGCAagtaaaggagaagaacttttc
7	A**GA**AATAATGACATTGAA**G**CT**G**CCAGCCCATCTCAACTTCAACATGAAGCAagtaaaggagaagaacttttc
8	A**GA**AATAATGACATTGAA**G**CT**G**CC**GC**CCCA**G**CTCAACTTCAACATGAAGCAagtaaaggagaagaacttttc
9	GTATTATTTGTTCTAGTTTACAAGTTTTAGTGCATCTTTGAGGCTTggatggcggcgttagtatc

DNA homologous to *PHO84* locus is shown in capital letters and mutations are printed in bold letters and underlined.
Plasmid complementary sequences are shown in lower-case letters.

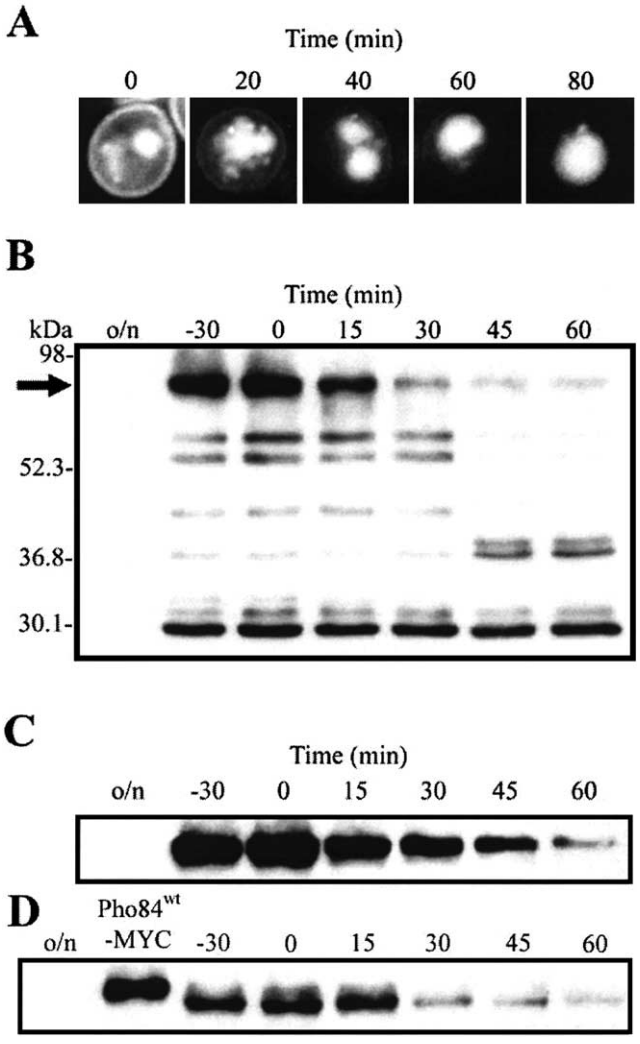

Fig. 1. Re-localization (A), and degradation of (B) Pho84^{WT}-GFP, (C) Pho84^{WT}-MYC, and (D) Pho84^{-18aa}-MYC proteins as a consequence of addition of repressible amounts of P_i. A: Fluorescence micrographs of LP_i defined media-grown JP11 cells expressing the complete Pho84 protein tagged with GFP. To the cell cultures repressible amounts of P_i (25 mM) were added at 0 min, and aliquots were withdrawn at 0, 20, 40, 60 and 80 min for fluorescence analyses. B: JP11/*PHO84^{WT}-GFP* cells were grown in YPD medium for 12 h (o/n), followed by continued growth in defined LP_i medium to an A_{600} of 2.0 (−30). P_i (10 mM) was added to cells at time zero (0), samples were taken at the indicated time points and spheroplasts (25 μg of protein) of the treated Pho84^{WT}-GFP protein-containing cells were prepared, followed by SDS–PAGE and immunoblotting as described in Section 2. The positions of the Pho84-GFP protein bands indicated by an arrow were compared with the migration of molecular mass standards. C: CEN.PK113-5D/*PHO84^{WT}-MYC* and (D) CEN.PK113-5D/*PHO84^{-18aa}-MYC* cells grown in YPD medium for 12 h (o/n), followed by continued growth in LP_i medium to an A_{600} of 2.5 (−30). After additional growth for 30 min (0) cells were treated with 10 mM P_i, for time periods (0–60) in min specified across the top of the panel. Samples were taken at the indicated time points and spheroplasts (25 μg of protein) of the treated Pho84^{WT}-MYC and Pho84^{-18aa}-MYC protein-containing cells were prepared, followed by SDS–PAGE and immunoblotting as described in Section 2. In panel D, a sample of Pho84^{WT}-MYC is included as a molecular size marker.

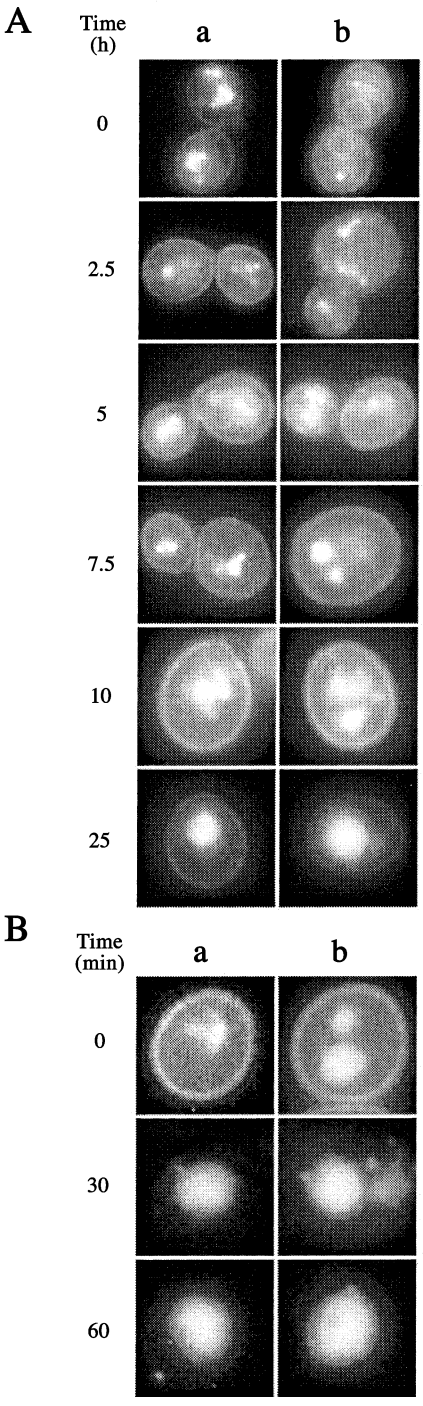

Fig. 2. Time-course study of localization of expressed wild-type and mutant Pho84-GFP proteins. A: Fluorescent micrographs of LP_i defined media-grown CEN.PK113-7D cells expressing (a) wild-type Pho84^{WT}-GFP and (b) mutated Pho84$^{KR,\,4SA}$-GFP harboring the following site-directed substitutions: K571R, S577A, S578A, S579A and S581A in the Pho84 protein. Images were analyzed and qualitatively compared at specified time points of growth up to 25 h. B: Repressible concentrations of P_i (25 mM) were added to CEN.PK113-7D cells grown in defined LP_i medium to an A_{600} of 3.7 corresponding to maximal expression of (a) wild-type Pho84^{WT}-GFP or (b) mutant Pho84$^{KR,\,4SA}$-GFP (K571R, S577A, S578A, S579A and S581A). Expression and internalization of the proteins were analyzed by fluorescent micrographs over a time course of 60min.

Table 2
Sequence of the cytoplasmic tails of Ste2, and Pho84 and mutants thereof

Ste2	319-fypgtlssfqtd**SINNDAKSS**lrsrlydly-348
Pho84^{WT}	558-hdeidpatlnfr**nkNNDieSS**spsqlqheaStop
Pho84^{WT}-MYC	558-hdeidpatlnfr**nkNNDieSS**spsqlqhea-MYC
Pho84^{-18aa}-MYC	558-hdeidpatlnfr-MYC
Pho84^{WT}-GFP	558-hdeidpatlnfr**nkNNDieSS**spsqlqhea-GFP
Pho84^{-18aa}-GFP	558-hdeidpatlnfr-GFP
Pho84^{KR}-GFP	558-hdeidpatlnfr**nrNNDieSS**spsqlqhea-GFP
Pho84^{4SA}-GFP	558-hdeidpatlnfr**nkNNDieAA**apaqlqhea-GFP
Pho84^{KR,2SA}-GFP	558-hdeidpatlnfr**nrNNDieAA**spsqlqhea-GFP
Pho84^{KR,4SA}-GFP	558-hdeidpatlnfr**nrNNDieAA**apaqlqhea-GFP

The SINNDAKSS sequence is highlighted and fully conserved residues are shown with capital letters. Arrows indicate the positions of the lysines in the Ste2 (upper) and Pho84 (lower) sequences. Site-directed mutations in the Pho84 are underlined. C-terminal additions of the MYC epitope or the GFP moiety are shown with boxes.

rescence and immunoblot analyses, it is likely that the 30 kDa GFP domain, which is stable over a broad pH range (reviewed in [25]), is not degraded as rapidly as the Pho84 part. Further support for this stability is the immunodetection of GFP shown in Fig. 1B, where the molecule is also present prior to the addition of repressible amounts of P_i, reflecting earlier turnover of the Pho84 protein.

As the GFP protein used in the Pho84^{WT}-GFP fusion protein is a large molecule it cannot be excluded that it may render structural and functional perturbations on the protein in the membrane and during the internalization and degradation process. Taking these considerations together with the high stability of this reporter tag, we wanted to eliminate any possible alterations that the GFP might have on the native Pho84 protein by the use of an alternative immunological reporter in the genomically expressed Pho84^{WT}-MYC chimera which is greatly reduced in size. Like the Pho84^{WT}-GFP fusion protein, the Pho84^{WT}-MYC (Fig. 1C) and Pho84^{-18aa}-MYC (Fig. 1D) proteins exhibit a similar sensitivity to P_i, with an almost fully depleted immunological detection at 60 min. The comparison between the chimeras revealed that the hydrophilic GFP reporter molecule, in spite of its size, did not confer an altered response to signaling and degradation as compared to the MYC-tagged Pho84 protein. It is also clear that these two chimeric constructs are suitable tools to study

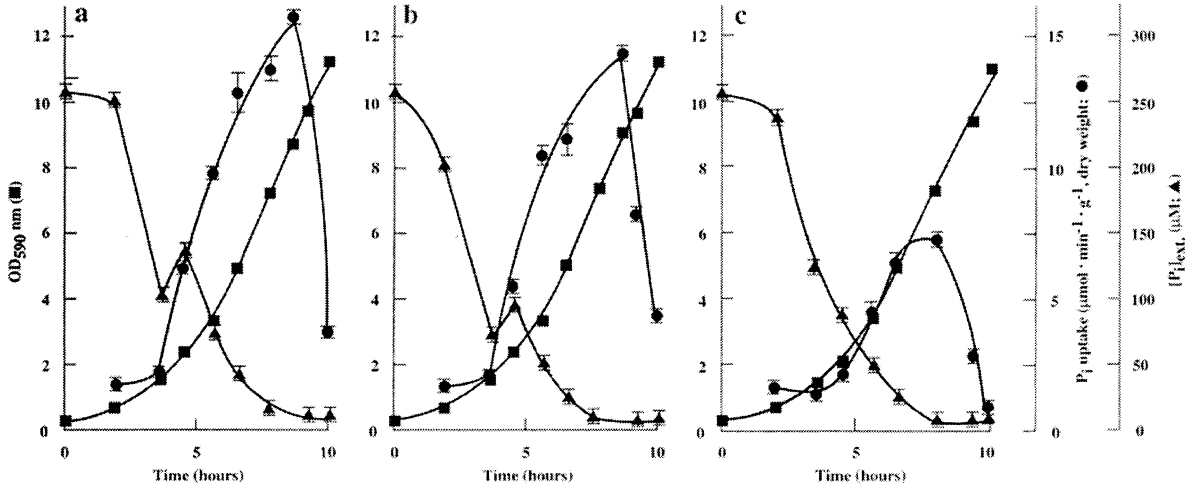

Fig. 3. Growth, phosphate uptake and extracellular phosphate determination for CEN.PK113-5D cells expressing (a) wild-type Pho84^{WT}; (b) Pho84^{WT}-MYC; and (c) truncated Pho84^{-18aa}-MYC proteins. Cells were grown in LP_i (■) medium. At specified time intervals, LP_i-grown cells were collected and assayed for inorganic phosphate uptake (●) and the LP_i supernatants were used for phosphate determination (▲) as described in Section 2. Values are the average of three determinations using the same cell preparation; bars indicate S.D. Three to four independent experiments were carried out.

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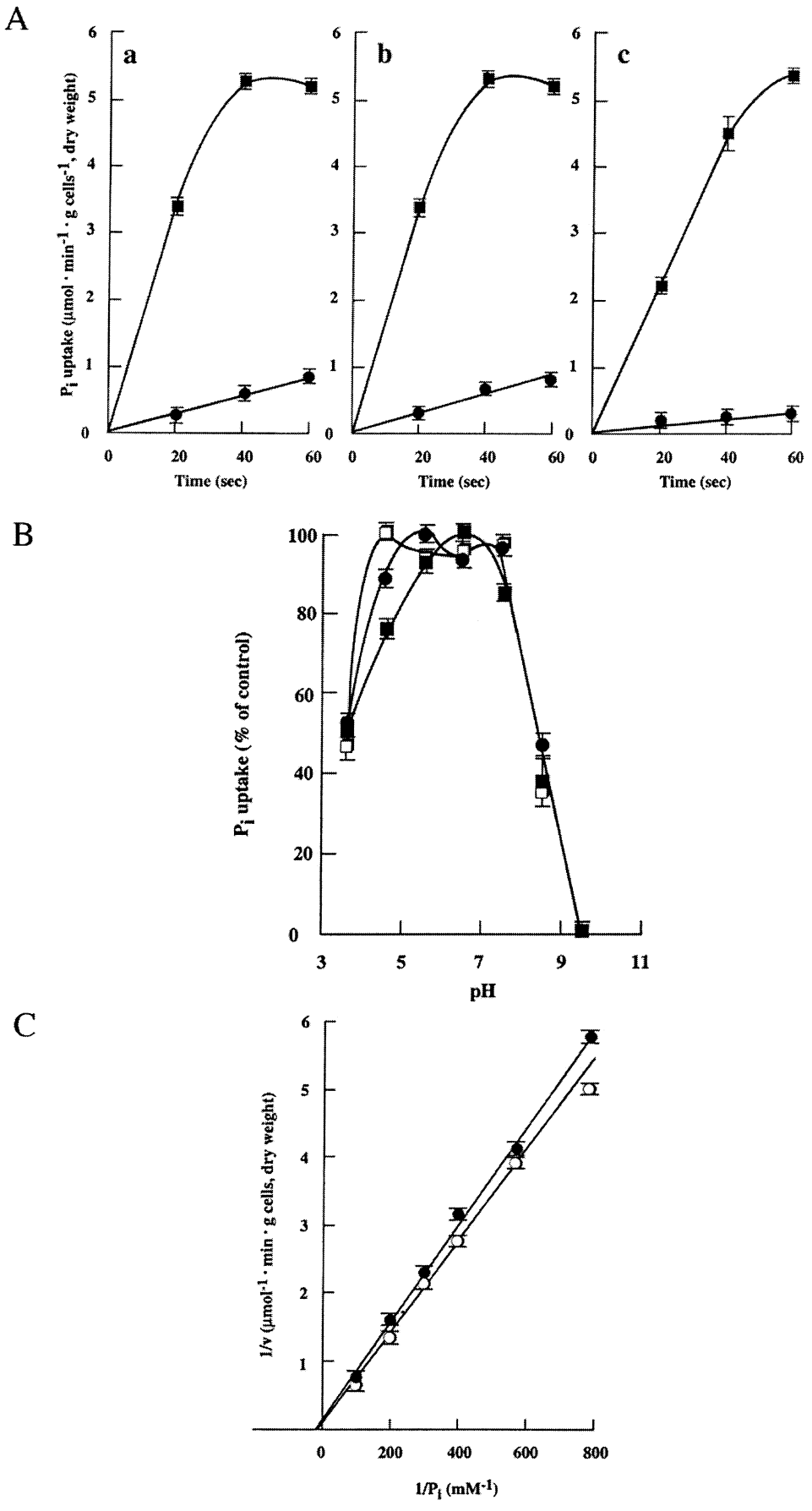
Fig. 4. Uncoupler sensitivity (A), pH-dependence (B), and kinetic characterization (C) of CEN.PK113-5D cells expressing (a) wild-type Pho84^{WT}, (b) Pho84^{WT}-MYC or (c) truncated Pho84^{-18aa}-MYC proteins. LP_i-grown cells were harvested at an A_{600} of 6.5–8.0, where the P_i uptake activity was maximal. A: Time courses of phosphate transport by LP_i-grown cells expressing (a) wild-type Pho84^{WT}, (b) Pho84^{WT}-MYC, or (c) truncated Pho84^{-18aa}-MYC proteins were analyzed at pH 5.5 in the absence (■) and in the presence (●) of CCCP. B: P_i transport activities of wild-type Pho84^{WT} (●), Pho84^{WT}-MYC (■) and truncated Pho84^{-18}-MYC (□) expressed in LP_i-grown cells were measured during the first 20 s of uptake, over a pH range of 3.5–9.5 in 25 mM assay buffer containing 3% glucose and 0.11 mM P_i. The linear rate of P_i uptake during the first 20 s was normalized with the individual highest uptake rate set to 100%. C: Lineweaver–Burke plots of phosphate transport by cells expressing wild-type (○) and truncated Pho84-MYC (●) proteins were analyzed for the first 20 s at pH 5.5 as a function of various external phosphate concentrations (1.26–11 μM). Values are the average of three determinations using the same cell preparation; bars indicate S.D. Three to four independent experiments were carried out.


A
a
b
c
P_i uptake (μmol · min^{-1} · g $cells^{-1}$, dry weight)
Time (sec)
B
P_i uptake (% of control)
pH
C
1/v (μmol^{-1} · min · g cells, dry weight)
1/P_i (mM^{-1})

discrepancies in internalization and degradation between native and modified Pho84 proteins.

3.2. The SINNDAKSS-like sequence of the Pho84 protein

We have identified a partly conserved SINNDAKSS-like sequence (nkNNDieSS) in the C-terminal tail of the Pho84 protein (Table 2) and assessed the potential role of the lysine and serine residues within and close to the SINNDAKSS-like sequence by construction of one truncated and four site-directed amino acid replacement mutants tagged with GFP (Table 2), and investigated their cellular localization during expression of the proteins and their sensitivity to exposure of repressive amounts of extracellular P_i through fluorescence microscopy. As illustrated in Fig. 2, where we compared the time dependence of localization of the Pho84^{WT}-GFP and Pho84^{KR,4SA}-GFP proteins, no significant difference was observed throughout the time course studied. The same pattern was seen for the strains expressing Pho84^{-18aa}-GFP, Pho84^{KR}-GFP, Pho84^{4SA}-GFP and PHO84$^{KR,\ 2SA}$-GFP proteins (not shown). Expression and localization (Fig. 2A,B) of these mutants could not be distinguished from that of the Pho84^{WT}-GFP protein. Therefore, the last 18-amino-acid sequence containing the nkNNDieSS sequence and the four point mutations therein, within the C-terminal tail appear not to be implicated in the correct targeting of the Pho84 protein to the plasma membrane. Perhaps of greater interest is the fact that the degradation process displays a similar sensitivity upon exposure of repressive amounts of external P_i, for all the mutants and Pho84^{WT}-GFP. Hence, we conclude that the C-terminal 'Ste2 SINNDAKS-like' sequence of the Pho84 transporter is not essential for modification of the protein preceding the internalization process of the protein.

3.3. Activity of C-terminally truncated Pho84 protein

Cells expressing Pho84^{WT}, Pho84^{WT}-MYC and Pho84^{-18aa}-MYC (Table 2) were grown in LP_i media for up to 10 h, external P_i concentration was monitored and withdrawn cells were assayed for high-affinity P_i uptake (Fig. 3). Repressed levels of P_i uptake during the lag phase were typically 1.7 μmol min^{-1} (g dry weight of cells)$^{-1}$, while maximally derepressed P_i uptake activities in the late exponential growth phases were 16 μmol min^{-1} (g dry weight of cells)$^{-1}$ for Pho84^{WT} protein (Fig. 3a), 14 μmol min^{-1} (g dry weight of cells)$^{-1}$ for the Pho84^{WT}-MYC protein (Fig. 3b), and 7.2 μmol min^{-1} (g dry weight of cells)$^{-1}$ for the Pho84^{-18aa}-MYC protein (Fig. 3c). After reaching this maximal uptake, the transport activity rapidly declined for both the chimeric protein constructs and Pho84^{WT}, an observation fully in agreement with our previous characterization of the Pho84 protein [6,10]. This rapid decline in transport activity is the result of the loss of elevated derepressed expression of the protein in the plasma membrane and the removal of existing proteins to the vacuole for degradation. The transient increase in extracellular P_i observed in Fig. 3a,b, in phase with the onset of the activation of the transporters, has been reported earlier [6]. To reach an understanding of the apparent differences between the Pho84^{WT}, Pho84-MYC and Pho84^{-18aa}-MYC proteins we analyzed the kinetics of the transporters. The H^+-dependence of the transport was analyzed in the absence and presence of the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP; Fig. 4A). It was shown that the P_i transport activity of the Pho84^{-18aa}-MYC protein was close to 50% of that seen with the Pho84^{WT} and Pho84-MYC proteins and that all three constructs exhibited a similar H^+-dependence for P_i transport of at least 90%. Next, the pH-dependencies of P_i uptake catalyzed by all three constructs were compared over a pH range of 3.5–9.5 (Fig. 4B). The proteins exhibited a high and relatively unchanged activity between pH 5.5 and 7.0. At pH values higher than 7.5, the activity rapidly declined. Finally, the kinetics of the Pho84^{WT} and Pho84^{-18aa}-MYC proteins in terms of K_m was addressed at P_i concentrations up to 11 μM in order to minimize the influence of the kinetics of the low-affinity transporter active at higher P_i concentrations (Fig. 4C). The Lineweaver–Burke plot shown is based on the initial P_i uptake rate as a function of P_i concentration as assayed for Pho84^{WT} and Pho84^{-18aa}-MYC proteins expressing cells grown in LP_i medium and harvested at identical A_{600} values. The apparent K_m values determined by linear regression analysis of obtained data were close to identical ($K_m^{WT} = 42.7$ μM, $K_m^{-18aa} = 42.2$ μM). Based on the above criteria it was concluded that both proteins are functional with regard to P_i binding and transport, although the catalytic efficiency of the Pho84^{-18aa}-MYC protein is lowered. The observation that the uptake activity of cells harboring Pho84^{-18aa}-MYC is reduced to about half of the full-length proteins, Pho84^{WT} and Pho84^{WT}-MYC (Fig. 3) suggest that the last 18 residues in the C-terminal tail of the protein are important but not essential for transport activity.

Understanding of the mechanisms for HP_i-stimulated post-transcriptional down-regulation of Pho84 will be an important step for elucidating the mechanism by which organisms modulate the accumulation of P_i and other nutrients. Identifying the amino acid residues in Pho84 that are required for this process will provide insights into how eukaryotes such as yeasts regulate intracellular P_i levels. Insights into the mechanism will, moreover, be provided by structure-function analyses of the Pho84 protein identifying the residues that are important for the translocation of P_i into the cell. The results of the present study suggest that the Pho84 C-terminal SINNDAKSS-like sequence, unlike the SINNDAKSS sequence of the Ste2 protein, is not a target site for signaling endocytosis. Additional studies will be required to further elucidate the identity and role of the residues taking part in this mechanism.

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