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**YEAST CHITIN SYNTHASE 2 ACTIVITY IS MODULATED BY PROTEOLYSIS AND
PHOSPHORYLATION**

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***Saccharomyces cerevisiae* chitin synthase 2 (Chs2) synthesizes the primary septum after mitosis is completed. It is essential for proper cell separation and expected to be highly regulated. We have expressed Chs2 and a mutant lacking the N-terminal region in *Pichia pastoris* in an active form at high levels. Both constructs show a pH and cation dependence similar to the wild-type enzyme, as well as increased activity after trypsin treatment. Using further biochemical analysis, we have identified two mechanisms of chitin synthase regulation. First, it is hyperactivated by a soluble yeast protease. This protease is expressed during logarithmic growth phase, when budding cells require Chs2 activity. Secondly, LC-MS/MS experiments on purified Chs2 identify twelve phosphorylation sites, all in the N-terminal domain. Four of them show the perfect sequence motif for phosphorylation by the cyclin-dependent kinase Cdk1. As we also show that phosphorylation of the N-terminal domain is important for Chs2 stability, these sites might play an important role in the cell cycle-dependent degradation of the enzyme, and thus in cell division.**

Chitin, a β -1,4-linked polymer of *N*-acetylglucosamine, is a polysaccharide that has an important structural role in many organisms, such as arthropods, molluscs, cephalopods, and fungi. The amounts of chitin present vary between these organisms; in yeast it constitutes only one percent of the cell wall, but still is an essential component [1, 2]. Chitin is synthesized from UDP-GlcNAc by chitin synthases (EC 2.4.1.16). Typically, these enzymes consist of three domains [3]: a hydrophilic amino-terminal domain, a highly conserved central catalytic domain and a hydrophobic carboxy-terminal domain integrated into the membrane (Fig. 1). Chitin synthases are classified into glycosyl transferase family 2 [4, 5], a sequence family that contains a wide range of enzymes. Besides chitin synthases, the most notable examples are the synthases of the structural polysaccharides cellulose, hyaluronan, β -1,3-glucan and β -1,4-mannan. The catalytic domains of the glycosyl transferase family 2 share a GT-A fold [6].

Saccharomyces cerevisiae contains three membrane-bound chitin synthases. Each enzyme has its own specific function: Chs1 has a repair function during cell separation [7], Chs2 is essential for primary septum formation and thus for cell division [8], and Chs3 synthesizes the chitin ring at bud emergence, as well as the chitin in the cell wall [2]. Since each chitin synthase acts at a different stage of the cell cycle, independent mechanisms for their regulation must exist in the cell. Disruption of specific chitin synthase genes also results in different effects. *S. cerevisiae* cells lacking Chs1 grow normally, without showing apparent loss of cell wall chitin content [9]. Likewise, disruption of the Chs3 gene was not lethal [10]. Therefore, these enzymes do not seem to be critical for yeast cell survival. On the other hand, disruption of Chs2 causes severe growth defects and morphological abnormalities [11, 12]. The regulation of Chs2 is different from that of the other two: its concentration in the cell changes greatly during the cell cycle, in contrast to the constant levels of Chs1 and Chs3 [13-15]. So, whereas regulation of Chs1 and Chs3 can only be posttranslational, Chs2 is also significantly regulated by synthesis and degradation.

Chs2 is especially interesting as it shows 55% sequence identity and is functionally analogous to chitin synthase 1 from the human pathogen *Candida albicans*, which is essential for cell survival [12]. The importance of these enzymes for their organisms, together with the lack of chitin synthases in humans, makes them excellent targets for antifungal drugs.

In this paper we report the heterologous expression and characterization of Chs2. As it had been previously shown that the N-terminal 222 amino acid residues of Chs2 could be truncated without loss of activity or function [16], we have also overexpressed the active mutant Chs2 Δ N222. Biochemical characterization of both proteins allows further insights in Chs2 regulation by posttranslational modification, as well as the role of the N-terminal domain.

Experimental procedures

Bacterial and yeast constructs. Genomic DNA from *S. cerevisiae* strain Y3437 was used for PCR amplification of the Chs2 and Chs2 Δ N222 genes. The primers used for amplifying Chs2 were: 5'-ATTGCACATATGACGAGAAACCCGTTTATGGTGG-3' and 5'-TCCTCGAGGCCCTTTTTGTGGAAAACATTTG-3', containing NdeI and XhoI sites respectively (underlined). For amplification of Chs2 Δ N222 the primers were: 5'-ATTGCACATATGGTCTCAGACTTGCCCTCC-3' and 5'-TCCTCGAGGCCCTTTTGTGGAAAACATTTG-3', containing NdeI and XhoI sites respectively (underlined). For bacterial expression, the two constructs were inserted into the NdeI and XhoI sites of the pET26b vector (Novagen). For expression in *P. pastoris*, the constructs were cloned in a modified pPICZ B vector (Invitrogen) containing a Strep 2 tag, prescission cleavage site, multiple cloning site for NdeI and XhoI restriction sites, prescission cleavage site, and His₁₀ tag; for this system expression is under the control of the alcohol oxidase (AOX1) promoter. All constructs contain a poly-histidine tag for protein detection by Western blot analysis. The correct insertion and sequence of each construct was confirmed by DNA sequencing.

***P. pastoris* culture conditions.** The protease-deficient *P. pastoris* strain SMD1163 (Invitrogen) was used for expression of the recombinant genes. The higher expressing clones were selected according to [17] and made into His⁺ mutants by transformation with a pPIC3.5 vector. For expression, SMD1163 cells carrying the foreign gene were grown overnight in MGY medium (0.34 % (w/v) yeast nitrogen base, 1 % (w/v) ammonium sulphate, 1 % (w/v) glycerol, 4 x 10⁻⁵ % (w/v) biotin) to an OD₆₀₀ nm of 2–6. The cells were centrifuged at 1,500 x g for 10 min, then suspended to an OD₆₀₀ of 1–2 in MM (0.34 % (w/v) yeast nitrogen base, 1 % (w/v) ammonium sulphate, 1.64 μ M biotin, 0.5 % (v/v) methanol) and grown for 24 h at 30 °C. Cells were harvested by centrifugation at 1,500 x g for 10 min and the pellets stored at -80 °C or used immediately for further studies.

***P. pastoris* membrane preparation.** Cells were suspended to an OD₆₀₀ of 50–100 in 30 mM HEPES pH 7.5 and 100 mM NaCl. After addition of an equal volume of ice-cold acid-washed glass beads (0.5 mm diameter), the cells were broken by vortex mixing for eight 30 s bursts separated by 30 s cooling on ice. Glass beads, cell debris and unbroken cells were removed by centrifugation at 1,500 x g for 10 min and the pellet was washed with an equal volume of buffer and centrifuged again. To separate the membrane fraction from the soluble components, the combined supernatants were then centrifuged at 100,000 x g for 30 min. The crude membrane pellet was suspended in 30 mM Tris or HEPES buffer pH 7.0, 7.5 or 8.0 and the protein content determined using the DC protein assay (BioRad).

Bacterial culture conditions. Chs2 and Chs2 Δ N222 were expressed in *E. coli* C41 (Avidis). Bacterial cells were grown at 37 °C in TB medium until OD₆₀₀ reached 0.6-0.8, and then induced with 2 mM IPTG for 3-5 h. Cells were harvested by centrifugation at 3,300 g for 15 min and used immediately for membrane preparation.

Bacterial membrane preparation. Cells were suspended in 30 mM Tris pH 7.5 and 150 mM NaCl. The cells were then passed three times through a Microfluidiser model M-110L (Microfluidics Corp., Newton, MA) equipped with a 110 μ m interaction chamber and a cooling coil, which were immersed in water at 0 °C. After centrifugation at 15,000 g for 10 min, the supernatant was centrifuged at 100,000 g for one hour. The pellet was suspended in 30 mM Tris pH 7.5, and the protein content was determined as above.

S. cerevisiae culture conditions. *S. cerevisiae* wild type strain YPH419 were grown overnight in YPD medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose) to OD₆₀₀ 0.5 (logarithmic phase) or to OD₆₀₀ 14 (stationary phase). The *P. pastoris* membrane preparation protocol was followed, and the supernatant of the 100,000 x g centrifugation step (soluble fraction) was stored at -80 °C or used immediately.

Activity assay. The assay for measuring chitin synthase activity was carried out according to a previously described method [18, 19] in a 50 μ l reaction mixture. Unless otherwise indicated, it contained 30 mM Tris pH 7.5, 5 mM MgCl₂ (for Chs2 Δ N222) or 5 mM MnCl₂ (for Chs2), 32 mM GlcNAc, 1 mM UDP-(U-¹⁴C)-GlcNAc (Amersham, specific activity 553,000 dpm/ μ mol), and 25 μ g of *P. pastoris* or 800 μ g of *E. coli* membranes. For investigation of the activity at a pH range of 6 to 9, a mixture of buffers was used. This mixture contained 30 mM HEPES, 30 mM MES and 30mM TAPS. To test divalent cation specificity, 5 mM of a cation salt (MgCl₂, MnCl₂, CoCl₂, NiCl₂ or CdCl₂) or 5 mM EDTA was used in the reaction mixture. Samples were incubated at 30 °C for 30 min for *P. pastoris*, or 16 to 20 h for *E. coli* membranes. To measure background activity from the chitin synthases of *P. pastoris*, membranes that did not contain overexpressed chitin synthases were used. Mean values and errors (standard deviation) were calculated from two sets of three independent experiments, using the program OriginPro 7.5.

Chitin synthesis was stopped by the addition of 1 ml of ice-cooled 10 % trichloroacetic acid, and the insoluble chitin was collected by filtration through a GF/B glass-fibre disc (Whatman) previously soaked in 10 % trichloroacetic acid. The filters were subsequently washed three times with 1 ml ice-cooled 10 % trichloroacetic acid, and twice with 1 ml ice-cooled 66 % ethanol, according to [20]. Filters were then transferred to scintillation fluid (Rotiscint eco plus, Roth). Synthesized chitin was quantified by determining the radiation level of the filter using a scintillation counter TRI-CARB 1500 (Canberra-Packard).

Proteolytic activation. For activation of Chs2 and Chs2 Δ N222 by trypsination, *P. pastoris* membranes were incubated with trypsin (Sigma-Aldrich) for 10 min at 30 °C. As previously reported [18, 21], the optimal amount of trypsin needed to be ascertained for each batch of membranes. The optimal trypsin/total membranes ratio varied from 1:30 to 1:150. After the 10 min incubation, proteolysis was stopped by adding a two-fold excess

of soybean trypsin inhibitor (Fluka).

To activate Chs2 and Chs2 Δ N222 using the soluble *S. cerevisiae* fraction, the soluble fraction was incubated with trypsin for 10 min at 30 °C, after which a two-fold excess of trypsin inhibitor was added. This mixture was then added to the chitin synthase-expressing membranes and incubated for 10 min at 30 °C.

Inactivation of soluble S. cerevisiae fraction. The trypsin-activated soluble *S. cerevisiae* fraction was incubated with each of the following protease inhibitors: pefabloc (100 μ M), E-64 (15 μ M), leupeptin (400 μ M), benzamidin (100 μ M) and pepstatin A (20 μ M). After this, the mixture was added to Chs2-containing membranes and the activity assay was carried out.

Protein dephosphorylation. 100 μ g of *P. pastoris* membranes were incubated with 2 units of calf intestine alkaline phosphatase (Roche) for 16 h at 4 °C.

Partial protein purification. *P. pastoris* membranes containing overexpressed Chs2 were diluted in solubilization buffer (200 mM NaCl, 30 mM imidazole pH 7.5, 10 % glycerol (w/v), 1 % Fos-choline-14) plus protease inhibitors (0.1 mg/ml trypsin inhibitor, 1 mM benzamidine, 0.1 mM pefabloc) and incubated for one hour at 4 °C. The unsolubilized material was removed by centrifugation for one hour at 170,000 g and loaded onto a Ni-sepharose (Amersham) column. The column was washed with wash buffer (70 mM imidazole pH 7.5, 150 mM NaCl, 0.05 % Fos-choline-14) and eluted with buffer containing 300mM imidazole pH 7.5, 150 mM NaCl and 0.05 % Fos-choline-14.

SDS/PAGE: 10% resolving gels were prepared and overlaid with a 3% stacking gel. Before electrophoresis the proteins were denatured in sample buffer (60 mM Tris pH 6.8, 1.5% SDS, 10% glycerol, 0.005% bromphenol blue, 50 mM DTT).

Western blotting. After SDS/PAGE, proteins were transferred onto PVDF membrane (ImmobilonTM pore size 0.45 μ m, Millipore) using a semi-dry transfer apparatus (Trans-Blot[®]SD, Bio-Rad). For immuno-detection, proteins were probed with monoclonal anti-poly-histidine antibody (Sigma-Aldrich) and visualized with anti-mouse alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich), both antibodies were diluted 1:1,000 in 20 mM Tris pH 7.5 plus 150 mM NaCl.

Phosphorylation site mapping. The partially purified protein was resolved by SDS/PAGE analysis and the gel fraction containing Chs2 was cut out for phosphopeptide mapping. This analysis was done at the Center for Functional Genomics (University at Albany, Rensselaer, NY). For this purpose, the protein was in-gel tryptic digested and the resulting phosphopeptides were enriched by TiO₂ IMAC. LC-MS/MS was performed on the enriched fraction to identify phosphorylation sites, and on the entire sample to determine the sequence coverage.

Protein phosphorylation. 150 mg of *P. pastoris* membranes were incubated with 20 units of M phase Cdc2-cyclin B (NEB, recognition motif S/TP-x-K/R) for 30 min at 30 °C in a 30 μ l reaction mixture containing 200 μ M γ -³²P-ATP (Perkin-Elmer, 3 mCi/ μ mol) and protease inhibitors (0.1mg/ml trypsin inhibitor, 1 mM benzamidine, 0.1 mM pefabloc). After SDS/PAGE, phosphorylated protein was detected by

autoradiography.

Results and discussion

We established the heterologous expression of the *S. cerevisiae* Chs2 and the deletion mutant Chs2 Δ N222 in *P. pastoris* and *E. coli*. To further study the proteins, we isolated membranes from both expression systems. As observed by immunodetection (Fig. 2), the proteins migrate on SDS/PAGE with the molecular weights expected from their amino acid sequences (115 and 90 kDa for Chs2 and Chs2 Δ N222, respectively, including affinity tags), showing that neither Chs2 nor Chs2 Δ N222 are glycosylated. This is consistent with a Chs2 Δ N193 mutant expressed in *S. cerevisiae* that was shown not to be glycosylated [22], and is further confirmed by the observation that the proteins expressed in *E. coli*, which lacks the glycosylation machinery of yeast, have molecular weights comparable to the *P. pastoris*-expressed proteins (Fig. 2).

Chs2 and Chs2 Δ N222 expressed in both expression systems are active. However, the activities of *E. coli*-expressed proteins were significantly lower than those from *P. pastoris*. Longer incubation time and higher amounts of membranes were needed to measure similar levels of activity. Therefore, for further characterization only the *P. pastoris*-expressed proteins were used. *P. pastoris* is a yeast with a chitinous cell wall like *S. cerevisiae*, and indeed, *P. pastoris* membranes not expressing Chs2 show chitin synthase activity. This activity is, however, negligible compared to that of overexpressed Chs2 or Chs2 Δ N222 (Fig. 3).

To determine whether both Chs2 and Chs2 Δ N222 show enzymatic characteristics similar to the wild-type protein, their dependence on both pH and divalent cations was investigated (Fig. 3). The *P. pastoris*-expressed Chs2 and Chs2 Δ N222 activities are strictly dependent on divalent cations (Fig. 3b). For Chs2 we obtained the highest activity in the presence of Mn²⁺, and reduced activity with Mg²⁺ and Co²⁺. Chs2 Δ N222 showed high activity in the presence of Mn²⁺ and Mg²⁺, with a slight preference for Mg²⁺, and, like the full-length protein, less activity with Ni²⁺ and Co²⁺. For all constructs, upon adding EDTA or Cd²⁺ to the reaction mixture activity was lost.

This preference for Mn²⁺ or Mg²⁺ fits with X-ray structures of glycosyl transferases with the GT-A fold, which show that either of these two cations bind the two phosphate groups of the UDP-sugar substrate, and are thought to assist in cleaving the sugar-UDP bond [6, 23]. The decrease of activity using the various divalent cations matches the decrease of their ionic radii, and also corresponds with the cation selectivity of wild type Chs2 and *S. cerevisiae*-expressed mutants [18, 22].

Besides the cation dependence, our two constructs also share an optimum pH between 7.5 and 8.5 with the wild-type enzyme (Fig. 3a) [18, 24, 11, 22]. Together, these results show that in *P. pastoris* the protein is expressed in a functional form, having the characteristics of the wild type enzyme. Moreover, when treated with appropriate amounts of trypsin, both proteins show high levels of hyperactivation (Fig. 4); this too is consistent with the wild type enzyme [18, 16]. Interestingly, the two proteins exhibit different levels of proteolytic hyperactivation: 18-fold for Chs2 Δ N222 vs. 6-fold for Chs2 (Fig. 4). The increased activation level for Chs2 Δ N222 as compared to Chs2 implies that

the N-terminal domain obstructs trypsin activity, and that its removal could lead to a more exposed activation site.

Regulation of Chs2

Chs2 synthesizes the primary septum separating mother and daughter cell after contraction of the acto-myosin ring [25, 26], at a very specific time and position. This requires strong regulation of Chs2 activity. Most obviously, the expression and degradation of the enzyme are under tight control, resulting in a half-life of only 25 minutes [14]. Although the expression of Chs2 peaks during mitosis [13], the protein accumulates in the endoplasmic reticulum until mitotic exit, when it is transported to the mother-bud neck [27]. It already disappears from the neck eight minutes later [28, 27], to be degraded by the major vacuolar protease PEP4 [14].

In addition to this metabolic regulation, post-translational modification has been observed for Chs2. Chs2 is phosphorylated *in vitro* by, and thus a possible *in vivo* target of, the cyclin-dependent kinase Cdk1 in complex with the mitotic phase cyclin Clb2 [29, 30]. Also, the *in vitro* chitin synthase activity is increased markedly by proteolysis [18].

Proteolytic activation

There is a long-standing question about the putative zymogenic form of chitin synthases. For *S. cerevisiae*, the three chitin synthases are active in their unprocessed form. Trypsin has been reported to stimulate chitin synthase activity of Chs1 and Chs2 [31, 18], but not of Chs3 [24]. In the case of Chs1, the stimulation was 7- to 15-fold [31, 18], trypsin treatment of Chs2 shows around four times increased activity [18]. In the case of the insect *M. sexta*, trypsin does not activate chitin synthesis directly. However, trypsin was found to activate a soluble stimulatory factor in the midgut, which increased chitin synthesis by 25-30% [32]. For our *P. pastoris*-expressed constructs, we measured 6 times activation by trypsin for the full-length protein, and 18 times for the N-terminal deletion mutant (Fig. 4, panel 6).

Even though hyperactivation by proteolysis has been well established for Chs1 and Chs2, no endogenous protease that could take this role has been identified. Chs1 was reported to be activated by *S. cerevisiae* proteinase B [31, 33]. However, proteinase B did not influence Chs1 activity *in vivo* [34, 35], demonstrating that either the observed proteolytic stimulation is not physiologically relevant, or performed by another protease.

We tested the possibility of hyperactivation of Chs2 by factors present in the soluble fraction of *S. cerevisiae* cells, using cells with a logarithmic growth rate, harvested at an OD₆₀₀ of 0.5. We found that incubation with the soluble fraction of *S. cerevisiae* cells in itself does not influence Chs2 activity in *P. pastoris* membranes. However, when the soluble fraction is first treated with trypsin, and then after inhibition of trypsin is added to the *P. pastoris* membranes, it does increase Chs2 and Chs2 Δ N22 activity two- to four-fold (Fig. 4, panel 3).

A number of controls were necessary to confirm that indeed there was an activating factor in the soluble fraction. First, the trypsin-activated soluble fraction did not show any chitin synthase activity by itself (Fig. 4, panel 4), excluding the possibility that the higher activity is caused by trypsin-activated chitin synthase in the soluble fraction. Second, no hyperactivation was observed when trypsin was inhibited before being added to the membranes (Fig. 4, panel 5), showing that there was no residual

trypsin activity.

Since trypsin activity in the trypsinated soluble fraction has been inhibited prior to adding this mixture to the membranes, the activating factor can only be a part of the soluble *S. cerevisiae* fraction. Moreover, the need for a protease to trigger the stimulatory effect implies that the activating factor in the soluble fraction is a protein. To test whether this soluble protein could be, like trypsin, a protease, we investigated the effect of a range of protease inhibitors on the activated soluble *S. cerevisiae* fraction. Before addition to the Chs2-containing membranes, the trypsin-activated soluble *S. cerevisiae* fraction was incubated with each of the following protease inhibitors: pefabloc, E-64, leupeptin, benzamidin or pepstatin A. Indeed, the addition of leupeptin, a serine and cysteine protease inhibitor, almost completely abolished the stimulatory effect, while it did not affect the activity of Chs2 or Chs2 Δ N222 by itself (Fig. 5).

Identification and isolation of this yeast protease and genetic experiments as performed by [35] will be necessary to understand the physiological role of this phenomenon. However, some characteristics of the activating protease can already be defined. First, the inhibition of activation by leupeptin shows that the enzyme in question is a serine or cysteine protease. Second, activation was only observed when the soluble *S. cerevisiae* fraction was extracted from cells growing logarithmically, and not when it was extracted from cells growing in a stationary phase. Therefore, all proteases that are expressed constitutively, as for instance the vacuolar proteases, can be ruled out as the activating protease. Moreover, the timing of expression of this protease hints to the *in vivo* relevance of proteolytic activation of Chs2. The protease expression is during the logarithmic phase, when cells are dividing, which is consistent with the timing of Chs2 activity: the primary septum is synthesized after mitosis has completed [36]. Thus, this protease is expressed during the phase when Chs2 activity would peak.

Interestingly, a very similar type of proteolytic regulation is observed for chitin synthase from *M. sexta*, where the trypsin-activated serine protease CTLP1 was identified to interact with the chitin synthase [37]. Despite this similarity, the location and function of this insect chitin synthase are completely different to those of Chs2: the enzyme creates a protective matrix that lines the midgut of the larvae [38]. Similar modes of regulation for two chitin synthases from very different species, performing very different functions, suggest that this activation mechanism might be widespread.

Phosphorylation

Another fast and reversible way of enzyme regulation by post-translational modification is protein phosphorylation. To find out whether phosphorylation influences the activity of Chs2, we submitted *P. pastoris* membranes containing either Chs2 or Chs2 Δ N222 to dephosphorylation by the unspecific calf intestine alkaline phosphatase. Dephosphorylation of membranes containing Chs2, whether trypsin-treated or not, does not significantly affect chitin synthase activity (Fig. 6, panel 3 and 4). Therefore, Chs2 does not appear to contain a phosphorylation site that plays a direct role in catalysis. However, when phosphatase treatment is followed by trypsination, activity is almost completely lost (Fig. 6, panel 5), indicating degradation of the protein. These results show that active Chs2 does contain one or more phosphorylation sites. These might, as for glycogen phosphorylase [39], be involved in organization and stabilization of the subunit structure of the enzyme. Similar experiments with Chs2 Δ N222 show no effect after phosphatase treatment (Fig. 6, panel 5), indicating that the phosphate groups that affect

protein stability interact with or are located in the N-terminal domain.

Previous knowledge about phosphorylation of Chs2 is summarized in Fig. 1. So far, Ser82 and Ser86 are the only phosphorylation sites that have been identified for wild-type Chs2 [40]. Additionally, Chs2 has been shown to be an *in vitro* substrate of the cyclin-dependent kinase Cdk1 [29, 30]. To map the phosphorylation sites of Chs2, we analyzed the purified enzyme by mass spectrometry [41, 42]. For this type of analysis, microgram amounts of pure protein are required. The level of Chs2 expression in *P. pastoris* allowed for purification of sufficient amounts of protein. We were able to solubilize the overexpressed enzyme in Fos-choline-14 detergent, and established an IMAC purification protocol using the His₁₀ affinity tag. This single purification step significantly enriched the Chs2 content in the protein mixture, yielding ~50 % pure Chs2. The enzyme was further separated by SDS/PAGE (Fig. 7a) and could be enriched to 100 % purity for mapping of phosphorylation sites by mass spectrometry.

LC-MS/MS analysis identified twelve phosphorylation sites (Table 1), all in the N-terminal domain. The localization of the sites is consistent with our dephosphorylation experiments. Whereas Chs2 is affected by dephosphorylation, Chs2 Δ N222, having only one of the twelve sites present, is not. Consequently, we can conclude that dephosphorylation of the N-terminal domain sensitizes the enzyme towards trypsin. Trypsin is a highly unspecific protease, which cleaves a polypeptide chain after almost every exposed positively charged amino acid residue. An elevated sensitivity towards trypsin usually indicates a less compactly folded and thus less stable protein. Dephosphorylation of the N-terminal domain clearly exposes and destabilizes parts of Chs2 that are stable when this domain is phosphorylated or not present at all. This hints at a regulatory function for (de)phosphorylation of the N-terminal domain, as for example an involvement in the short lifetime of the enzyme.

Expression, localization and degradation of Chs2 are cell cycle dependent [15]. The enzyme is mainly expressed during mitosis [13]. It is transported from the endoplasmic reticulum to the mother-bud neck at mitotic exit, to synthesize the primary septum [27]. Finally, after cytokinesis it is proteolytically processed in the vacuole [14]. Such a complex cycle requires tight regulation of activity and stability. If phosphorylation is important for protein stability, the enzyme should be phosphorylated immediately after synthesis, and dephosphorylation could then facilitate rapid degradation of the protein when this becomes necessary.

In this light, it is interesting to note that four phosphorylation sites match the perfect phosphorylation motif (S/TP-x-K/R) for Cdk1, a cyclin-dependent kinase that controls many cell cycle events, and that they are all located in the N-terminal domain (Fig. 1). Furthermore, all four sites are observed in our LC-MS/MS experiments. Indeed, Chs2 has been revealed as an *in vitro* substrate of Cdk1, with the highest phosphorylation rate when it is complexed with Clb2 [29, 30]. We have used the homologous human complex in combination with γ -³²P-ATP and have shown that Chs2 Δ N222 is not phosphorylated by this complex while Chs2 is (Fig. 7b). Thus, Cdk1 phosphorylation by Cdk1 is at the N-terminal domain, which is consistent with the phosphorylation sites observed with MS/MS.

The N-terminal domains of chitin synthases can be quite different, and their regulatory role is uncertain [16]. However, CaChs1, the functional homologue of Chs2

from *C. albicans*, has an N-terminal domain that is homologous to that of Chs2, and contains three perfect Cdk1 phosphorylation motifs. Indeed, all N-terminal domains that are homologous to that of Chs2 (UniProtKB primary accession numbers Q6FQN5, A7TEI4, Q6CS65, Q758F2 and Q6BZ46) contain multiple perfect Cdk1 phosphorylation sites, in most cases even in the same position as for Chs2. Therefore, it appears that the Cdk1 phosphorylation sites are a feature of this family of N-terminal domains, strengthening the suggestion of a functional role.

Chs2 is most efficiently phosphorylated when Cdk1 is complexed with Clb2 [30]. As this is an M phase cyclin, it is active during mitosis, when Chs2 is synthesized, but not after cytokinesis, when Chs2 is degraded. Cdk1-Clb2 phosphorylates the N-terminal domain of Chs2, and the timing of its activity is correlated with that of Chs2. As tempting as it is to speculate about a possible role for Cdk1 in regulating Chs2 activity, site-directed mutagenesis studies will be necessary to confirm whether these specific phosphorylation sites are of importance for the short lifetime of Chs2.

Footnotes - The abbreviations used are: Chs, chitin synthase; GlcNAc, *N*-acetylglucosamine; MGY, minimal glycerol; OD, optical density; MM, minimal methanol; TB, terrific broth; IPTG, isopropyl β -D-1-thiogalactopyranoside; YPD, yeast extract peptone dextrose; LC, liquid chromatography; MS/MS, tandem mass spectrometry.

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Table 1. Mass spectrometry identification of phosphorylation sites on Chs2

4	NPFMVEPS*NGSPNR	17
4	NPFMVEPSNGS*PNR	17
36	WANPSEESLEDSYDQSNVFQGLPAS*PSR	63
64	AALRY <u>S</u> PDRR	73
93	YAANLQES*PKR	103
121	DNADLPVDPY*HLSPQQPSNNLFGSGR	147
121	DNADLPVDPYHLS*PQQPSNNLFGSGR	147
121	DNADLPVDPYHLSPQQPSNNLFGS*GR	147
155	YTMSTT <u>S</u> TTAPSLAEADDEKEK	176
177	YLTS*TTSYDDQSTIFSADTFNETK	200
177	YLTSTTSYDDQSTIFS*ADTFNETK	200
254	RN <u>S</u> PEFTEMR	263

The table lists the phospho-peptides that were identified based on neutral loss of a mass of 98 and three continuous ion series. The residues denoted with an asterisk (*) are phosphorylated residues identified without ambiguity. The underlined residues are sites identified with neutral loss of a mass of 98 and only two continuous ion series. Sequence coverage was 56%.

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Figure legends

Fig. 1. Schematic drawing of the domain organisation of Chs2. The N-terminal soluble domain and the central catalytic domain are located in the cytoplasm; the hydrophobic C-terminal domain is anchored in the plasma membrane. Shaded circles indicate the positions of observed phosphorylation sites [40], clear circles show those of the consensus phosphorylation sequences for cyclin-dependent kinase 1. Residue 222, the end of the region that is deleted in the Chs2 Δ N222 mutant, is indicated by a cross.

Fig. 2. Western blot analysis of membranes shows that in both expression systems the recombinant proteins were integrated into the membrane. Each protein migrates according to its sequence-based predicted molecular weight, and to the same level when expressed in yeast or bacteria, which implies that both are not glycosylated in *P. pastoris*. The His-tagged proteins were probed with monoclonal anti-poly-histidine antibody and visualized with alkaline phosphatase-conjugated secondary antibody. Per lane: **M**) Marker in kDa, **1**) Chs2 (115 kDa) in *P. pastoris*, **2**) Chs2 Δ N222 (90 kDa) in *P. pastoris* **3**) Chs2 in *E. coli*, **4**) Chs2 Δ N222 in *E. coli*.

Fig. 3. Biochemical characterization of *P. pastoris*-expressed Chs2 and Chs2 Δ N222 shows identical enzymatic properties as compared to the wild-type protein. Black and light-grey bars show the activities of Chs2 and Chs2 Δ N222, respectively. Dark-grey bars show background activity of native chitin synthases of *P. pastoris*. **a.** The dependence of protein activity on pH, in the presence of 5 mM MnCl₂. To ascertain that all differences measured were due only to pH, for each experiment the same buffer mixture (30 mM HEPES, 30 mM MES and 30 mM TAPS) was used. **b.** The effect of cations on protein activity, assayed at pH 7.5. Each bar comprises two independent sets of three experiments (n=6).

Fig. 4. Hyperactivation of *P. pastoris*-expressed Chs2 (black) and Chs2 Δ N222 (grey) by trypsin and/or activated soluble fraction of *S. cerevisiae*. Activities are shown relative to their corresponding **1**) base activities (non-treated membranes, 6.0 and 5.5 nmol GlcNAc incorporated for Chs2 and Chs2 Δ N222, respectively, each set to 100%). **2**) Activities of membranes in the presence of soluble *S. cerevisiae* fraction, **3**) activities of membranes in the presence of trypsin-activated soluble *S. cerevisiae* fraction, **4**) activities of membranes in the presence of inhibited trypsin, **5**) activities of trypsin-treated membranes, **6**) activity of trypsin-activated soluble *S. cerevisiae* fraction by itself. Each bar comprises two independent sets of three experiments (n=6).

Fig. 5. Activation of *P. pastoris*-expressed Chs2 and Chs2 Δ N222 by the *S. cerevisiae* soluble fraction is inhibited by leupeptin. Activity of Chs2 (black) and Chs2 Δ N222 (grey) is shown relative to **1**) base activity (non-treated membranes, set to 100%). **2**) Membranes treated with activated soluble *S. cerevisiae* fraction, **3**) activated soluble *S. cerevisiae* fraction inhibited by 400 μ M leupeptin before addition to membranes, **4**) membranes treated with 400 μ M leupeptin. Each bar comprises two independent sets of three experiments (n=6).

Fig. 6. Phosphatase treatment of *P. pastoris*-expressed Chs2 (black) and Chs2 Δ N222 (grey). Activities are shown relative to their corresponding **1**) base activities (non-treated membranes, 6.0 and 5.5 nmol GlcNAc incorporated for Chs2 and Chs2 Δ N222,

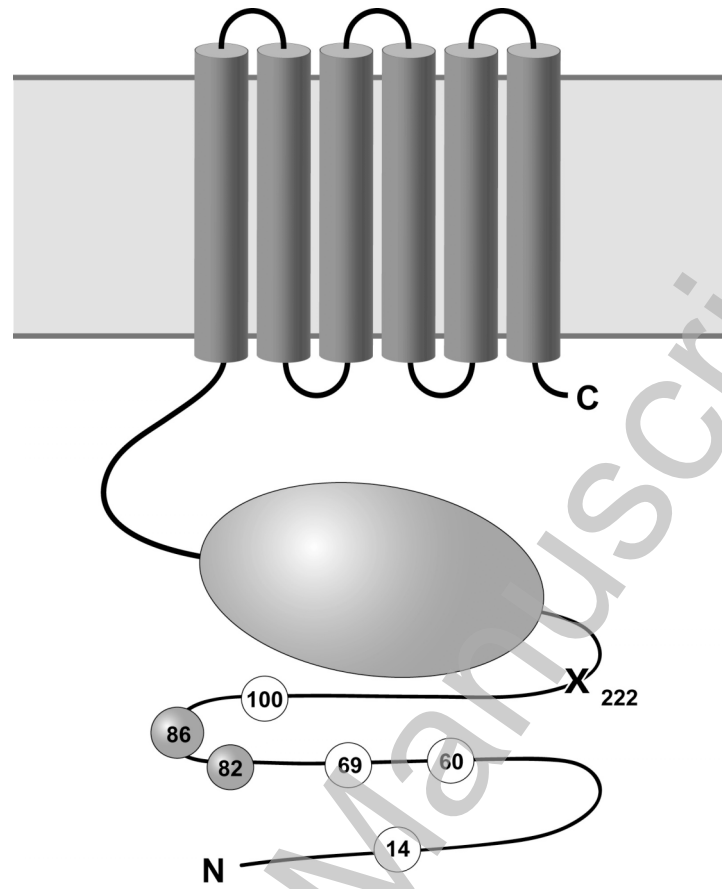
respectively, each set to 100%). **2)** Activities of trypsin-treated membranes, **3)** activities of membranes treated with alkaline phosphatase, **4)** activities of membranes treated with trypsin, followed by alkaline phosphatase **5)** activities of membranes treated with alkaline phosphatase, followed by trypsin. Each bar comprises two independent sets of three experiments (n=6).

Fig. 7. Phosphorylation analysis. **a.** SDS/PAGE analysis of partially purified Chs2. Left lane: marker in kDa, right lane: Chs2 (115 kDa) partially purified by IMAC. The single purification step followed by SDS/PAGE allowed for the isolation of pure Chs2 for LC-MS/MS phosphorylation site mapping. **b.** Autoradiography analysis of an SDS/PAGE gel from *P. pastoris* membranes containing overexpressed Chs2 or Chs2 Δ N222 after treatment with Cdc2-cyclin B plus γ -³²P-ATP. Per lane: M) Marker in kDa, 1) Membranes containing overexpressed Chs2 show a band at the molecular weight of the recombinant protein 2) Membranes containing overexpressed Chs2 Δ N222 do not show a signal at the molecular weight of the recombinant protein.

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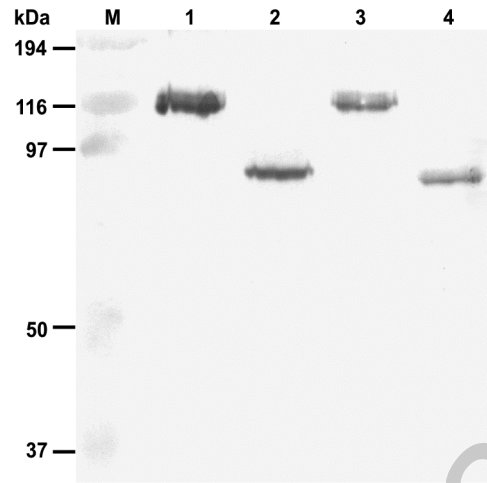
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Fig. 1



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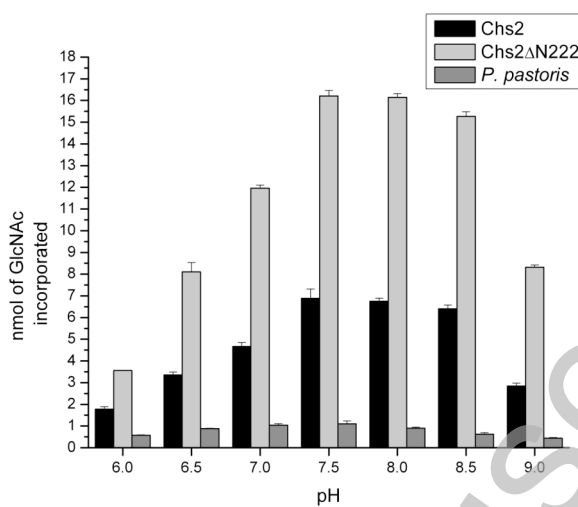
Fig. 2



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Fig. 3a



3b

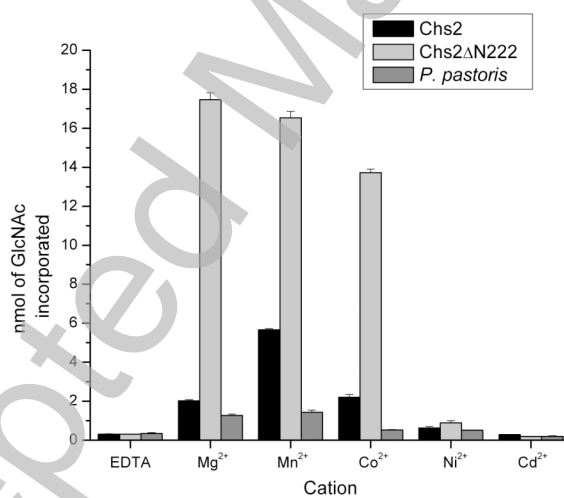
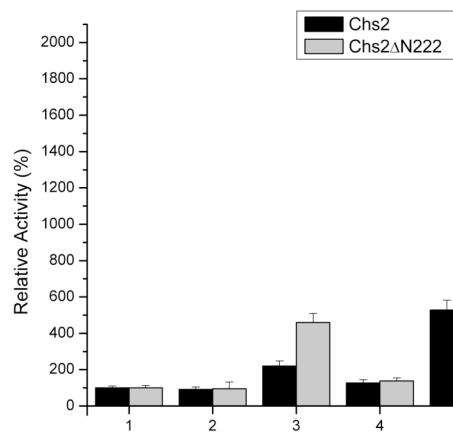


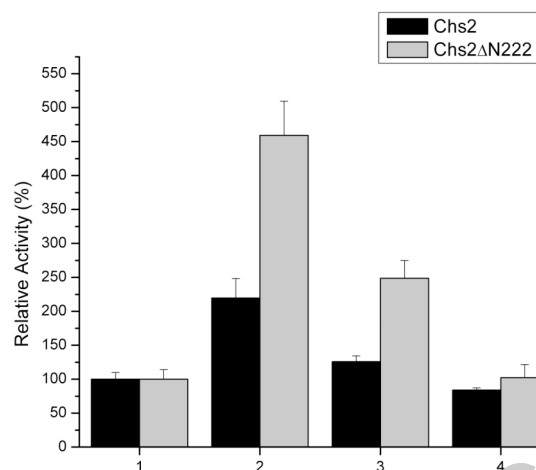
Fig. 4



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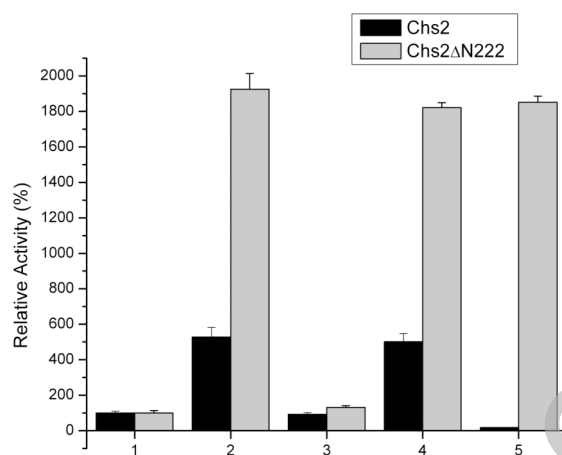
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Fig. 5



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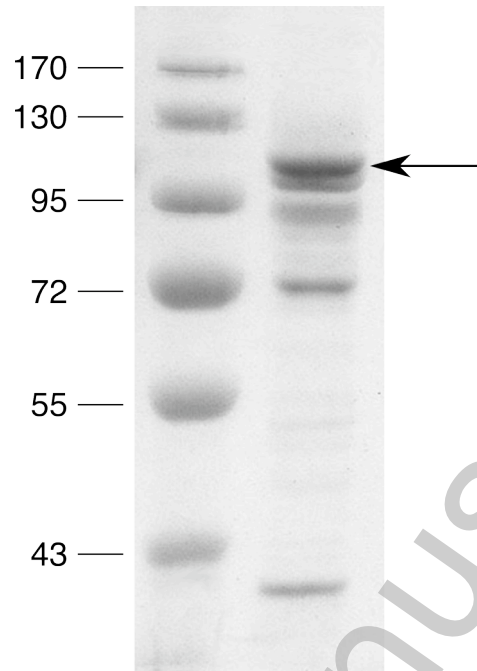
Fig. 6



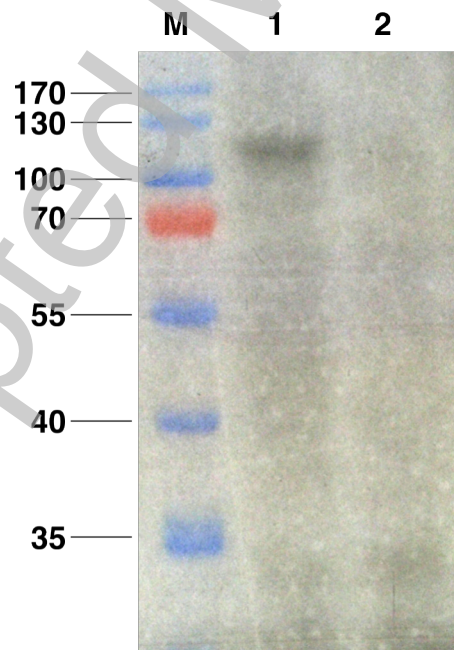
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Fig. 7a



7b



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