INTRODUCTION

Chitin, a β-1,4-linked polymer of N-acetyl-D-glucosamine, 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 1% of the cell wall, but still is an essential component [1,2]. Chitin is synthesized from UDP-GlcNAc (UDP-N-acetylglucosamine) by chitin synthases (EC 2.4.1.16). Typically, these enzymes consist of three domains [3]: a hydrophilic N-terminal domain, a highly conserved central catalytic domain and a hydrophobic carboxy-terminal domain integrated into the membrane (Figure 1). Chitin synthases are classified as part of the glycosyl transferase family 2 [4,5], which, like other members of the N-terminal 222 amino acid residues of Chs2 could be truncated for 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 exponential growth phase, when budding cells require Chs2 activity. Secondly, LC-MS/MS (liquid chromatography tandem MS) experiments on purified Chs2 identify 12 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.

Key words: chitin synthase, overexpression, regulation, phosphorylation, post-translational modification, proteolytic activation.

EXPERIMENTAL PROCEDURES

Bacterial and yeast constructs

Genomic DNA from S. cerevisiae strain Y3437 was used for PCR amplification of the Chs2 and Chs2\_N222 genes. The primers used for amplifying Chs2 were 5'-ATTGC\_CATATG\_ACGAGAAC\_CCGTTTAT\_GTTGG-3' and 5'-TCTC\_GAGG\_CCCTTTTT\_GGGAAA\_ACG\_TTTTG-3', containing NdeI and XhoI sites respectively (underlined). For amplification of Chs2\_N222, the primers were 5'-ATTGC\_CATATG\_TCTC\_ACG\_ACG\_ACG\_TCTC\_C\_3' and 5'-TCTC\_GAGG\_CCCTTTTT\_GTTG\_A\_ACG\_TTTTG\_3', containing NdeI and XhoI sites respectively (underlined). For bacterial expression, the two constructs were inserted into the pTrcHis vector (Novagen).

Saccharomyces cerevisiae Chs2 (chitin synthase 2) synthesizes the primary septum after mitosis is completed. It is essential for proper cell separation and is 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 exponential growth phase, when budding cells require Chs2 activity. Secondly, LC-MS/MS (liquid chromatography tandem MS) experiments on
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 (minimal glycerol) medium until the D600 had reached 0.6–0.8, and then induced with 2 mM IPTG (isopropyl-β-D-thiogalactoside) for 3–5 h. Cells were harvested by centrifugation at 33 000 g for 15 min and used immediately for membrane preparation.

Bacterial culture conditions

Chs2 and Chs2ΔN222 were expressed in Escherichia coli C41 (Avidis). Bacterial cells were grown at 37°C in TB (Terrific broth) medium until the D600 had reached 0.6–0.8, and then induced with 2 mM IPTG (isopropyl-β-D-thiogalactoside) for 3–5 h. Cells were harvested by centrifugation at 33 000 g for 15 min and used immediately for membrane preparation.

Bacterial membrane preparation

Cells were suspended in 30 mM Tris/HCl (pH 7.5) and 150 mM NaCl. The cells were then passed three times through a Microfluidiser model M-110L (Microfluidics, Newton, MA, U.S.A.) equipped with a 110 μm 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 1 h. The pellet was suspended in 30 mM Tris/HCl (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 D600 = 0.5 (exponential phase) or to D600 = 14 (stationary phase). The P. pastoris membrane preparation protocol was followed, and the supernatant of the 100 000 g centrifugation step (soluble fraction) was stored at −80°C or used immediately.

Activity assay

The assay for measuring chitin synthesis activity was carried out according to a previously described method [18,19] in a 50 μl reaction mixture. Unless otherwise indicated, it contained 30 mM Tris/HCl, pH 7.5, 5 mM MgCl2 (for Chs2ΔN222) or 5 mM MnCl2 (for Chs2), 32 mM GlcNAc, 1 mM UDP-[U-14C]-GlcNAc (Amersham, specific radioactivity 55 000 d.p.m./μmol), and 25 μg of P. pastoris or 800 μg of E. coli membranes. For investigation of the activity at a pH range of 6–9, a mixture of buffers was used. This mixture contained 30 mM Heps, 30 mM Mes and 30 mM Taps. To test bivalent 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 where incubated at 30°C for 30 min for P. pastoris, or 16–20 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-cold 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 of ice-cold 10% trichloroacetic acid, and twice with 1 ml of ice-cold 66% ethanol, according to [20]. Filters were then transferred to scintillation fluid and 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 g for 30 min. The crude membrane pellet was suspended in 30 mM Tris or Heps buffer, pH 7.0, 7.5 or 8.0, and the protein content determined using the DC protein assay (BioRad).

P. pastoris membrane preparation

Cells were suspended to a D600 of 50–100 in 30 mM Heps, 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 1500 g for 10 min and the pellet was washed with
(Rotiscint eco plus, Roth). Synthesized chitin was quantified by determining the radiation level of the filter using the scintillation counter TRI-CARB 1500 (Canberra-Packard).

Proteolytic activation

For activation of Chs2 and Chs2ΔN222 by trypsinization, \textit{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 2-fold excess of soybean trypsin inhibitor (Fluka).

To activate Chs2 and Chs2ΔN222 using the soluble \textit{S. cerevisiae} fraction, the soluble fraction was incubated with trypsin for 10 min at 30°C, after which a 2-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 \textit{S. cerevisiae} fraction

The trypsin-activated soluble \textit{S. cerevisiae} fraction was incubated with each of the following protease inhibitors: Pefabloc® (100 μM), E-64 (15 μM), leupeptin (400 μM), benzamidine (100 μM) or pepstatin A (20 μM). After this, the mixture was added to Chs2-containing membranes and the activity assay was carried out.

Protein dephosphorylation

\textit{P. pastoris} membranes (100 μg) were incubated with 2 units of calf intestine alkaline phosphatase (Roche) for 16 h at 4°C.

Partial protein purification

\textit{P. pastoris} membranes containing overexpressed Chs2 were diluted in solubilization buffer [200 mM NaCl, 30 mM imidazole pH 7.5, 10% (w/v) glycerol and 1% Fos-choline-14] plus protease inhibitors (0.1 mg/ml trypsin inhibitor, 1 mM benzamidine and 0.1 mM Pefabloc®) and incubated for 1 h at 4°C. The unsolubilized material was removed by centrifugation for 1 h at 170000 g and loaded on to a Ni-Sepharose (Amersham) column. The column was washed with wash buffer (70 mM imidazole, pH 7.5, 150 mM NaCl and 0.05% Fos-choline-14) and eluted with buffer containing 300 mM imidazole, pH 7.5, 150 mM NaCl and 0.05% Fos-choline-14.

SDS/PAGE

Resolving gels (10%) were prepared and overlaid with a 3% stacking gel. Before electrophoresis, the proteins were denatured in sample buffer (60 mM Tris/HCl, pH 6.8, 1.5% SDS, 10% glycerol, 0.005% Bromophenol Blue and 50 mM dithiothreitol).

Western blotting

After SDS/PAGE, proteins were transferred on to PVDF membrane (Immobilon™ pore size 0.45 μm, 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 (Anti-mouse alkaline phosphatase-conjugated secondary antibody: 1:500, Roche) and [γ-32P]ATP (Perkin-Elmer, 3 μCi/nl) at 30°C for 1 h. The autoradiogram was exposed to X-omat (Eastman Kodak). The autoradiogram was visualized with ALKALINE PHOSPHATASE-conjugated secondary antibody (1:500, Roche). The autoradiogram was visualized with ALKALINE PHOSPHATASE-conjugated secondary antibody (1:500, Roche).

RESULTS AND DISCUSSION

We established the heterologous expression of the \textit{S. cerevisiae} Chs2 and the deletion mutant Chs2ΔN222 in \textit{P. pastoris} and \textit{E. coli}. To study the proteins further, we isolated membranes from both expression systems. As observed by immunodetection (Figure 2), the proteins migrate on SDS/PAGE with the molecular masses expected from their amino acid sequences (115 and 90 kDa for Chs2 and Chs2ΔN222, respectively, including affinity tags), showing that neither Chs2 nor Chs2N222 is glycosylated. This is consistent with a Chs2ΔN193 mutant expressed in \textit{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 masses comparable with the *P. pastoris*-expressed proteins (Figure 2).

Chs2 and Chs2ΔN222 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 similar to *S. cerevisiae*, and indeed, *P. pastoris* membranes not expressing Chs2 show chitin synthase activity. This activity is, however, negligible compared with that of overexpressed Chs2 or Chs2ΔN222 (Figure 3).

To determine whether both Chs2 and Chs2ΔN222 show enzymatic characteristics similar to the wild-type protein, their dependece on both pH and bivalent cations was investigated (Figure 3). The *P. pastoris*-expressed Chs2 and Chs2ΔN222 activities are strictly dependent on bivalent cations (Figure 3B). For Chs2, we obtained the highest activity in the presence of Mn$^{2+}$, and reduced activity with Mg$^{2+}$ and Co$^{2+}$. Chs2ΔN222 showed high activity in the presence of Mn$^{2+}$ and Mg$^{2+}$, with a slight preference for Mg$^{2+}$, and, similar to the full-length protein, less activity with Ni$^{2+}$ and Co$^{2+}$. For all constructs, activity was lost upon addition of EDTA or Cd$^{2+}$ to the reaction mixture.

This preference for Mn$^{2+}$ or Mg$^{2+}$ 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 bivalent cations matches the decrease of their ionic radii, and also corresponds to 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 (Figure 3A) [11,18,22,24]. 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 (Figure 4); this too is consistent with the wild-type enzyme [16,18]. Interestingly, the two proteins exhibit different levels of proteolytic hyperactivation: 18-fold for Chs2ΔN222 compared with 6-fold for Chs2 (Figure 4). The increased activation level for Chs2ΔN222 as compared with 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 actomyosin 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 min [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 8 min later [27,28], 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 is 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 [18,31], but not of Chs3 [24]. In the case of Chs1, the stimulation was 7- to 15-fold [18,31], and trypsin treatment of Chs2 shows approx. 4-fold increased activity [18]. In the case of the insect Manduca 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 a 6-fold activation by trypsinization for the full-length protein, and 18-fold for the N-terminal deletion mutant (Figure 4, condition 5).

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 protease B [31,33]. However, protease B did not influence Chs1 activity in vivo [34,35], demonstrating that the observed proteolytic stimulation is either 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 exponential growth rate, harvested at a Dmax 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 Chs2ΔN222 activity 2- to 4-fold (Figure 4, condition 3).

A number of controls were necessary to confirm that there was indeed an activating factor in the soluble fraction. First, the trypsin-activated soluble fraction did not show any chitin synthase activity by itself (Figure 4, condition 6), 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 (Figure 4, condition 4), showing that there was no residual trypsin activity.

Since trypsin activity in the trypsinized 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, similar to 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, benzamidine 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 Chs2ΔN222 by itself (Figure 5).

Identification and isolation of this yeast protease and genetic experiments as performed in [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 exponentially, and not when it was extracted from cells growing in a stationary phase. Therefore, all proteases that are expressed constitutively, such as the vacuolar proteases, can be ruled out as the activating protease. Moreover, the timing of expression of this protease hints to the relevance in vivo of proteolytic activation of Chs2. The protease expression is during the exponential 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 Chs2ΔN-222 to dephosphorylation by the unspecific calf intestine alkaline

![Figure 5](image-url)
Figure 6 Phosphatase treatment of P. pastoris-expressed Chs2 (black) and Chs2ΔN222 (grey)

Activities are shown relative to their corresponding: (1) basal activities (non-treated membranes, 6.0 and 5.5 nmol of GlcNAc incorporated for Chs2 and Chs2ΔN222 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 ± S.D. (n = 6).

Phosphatase. Dephosphorylation of membranes containing Chs2, whether trypsin-treated or not, does not significantly affect chitin synthase activity (Figure 6, conditions 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 trypsinization, activity is almost completely lost (Figure 6, condition 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 Chs2ΔN222 show no effect after phosphatase treatment (Figure 6, condition 5), indicating the different products that affect protein stability interact with or are located in the N-terminal domain.

Previous knowledge about phosphorylation of Chs2 is summarized in Figure 1. So far, Ser62 and Ser66 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 analysed the purified enzyme by LC-MS/MS [41,42]. For this type of analysis, microgram amounts of pure Chs2 were needed (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, Chs2ΔN222, having only one of the 12 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

Table 1 LC-MS/MS identification of phosphorylation sites on Chs2

The Table lists the phosphopeptides 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 %.

Starting amino acid | Phosphorylation sequence |
--- | --- |
4 | NFMVEPS*NSPQR 17 |
4 | NFMVPSNS*PFRR 17 |
36 | WAPSEESLEDSYDGSNFQGLPS*PSR 63 |
64 | AALRSPDRR 73 |
93 | YAANQS*KPR 103 |
121 | DHAAPDPYHLS*PODQPSNGLFGSGR 147 |
121 | DNALPDPYHLS*PODQPSNGLFGSGR 147 |
121 | DNALPDPYHLS*PODQPSNGLFGSGR 147 |
155 | YTMSTSTPTAPSALADDEKEK 176 |
177 | YLTS*YTSYDQSTIFSADTNETK 200 |
177 | YLTSYDQSTIFS*ADTNETK 200 |
254 | RNSPEFTMR 263 |

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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/T-P-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 (Figure 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 Chs2ΔN222 is not phosphorylated by this complex, whereas Chs2 is (Figure 7B). Thus, phosphorylation by Cdk1 is at the N-terminal domain, which is consistent with the phosphorylation sites observed with LC-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.

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REFERENCES


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Characterization and regulation of yeast chitin synthase 2

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