Different roles for the stress-activated protein kinase pathway in the regulation of trehalose metabolism in *Schizosaccharomyces pombe*

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The Wis1p-Sty1p mitogen-activated protein kinase cascade is a major signalling system in the fission yeast *Schizosaccharomyces pombe* for a wide range of stress responses. It is known that trehalose functions as a protective metabolite to counteract deleterious effects of environmental stresses. Herein it is reported that the expression of genes related to trehalose metabolism in *S. pombe*, *ntp1*+ (neutral trehalase) and *tps1*+ [trehalose-6-phosphate (T6P) synthase], is partially regulated by the Sty1p kinase under salt-induced osmotic stress and conditions of slight oxidative stress and is fully dependent on this kinase under severe oxidative stress. This control is carried out through transcription factors Atf1p/Pcr1p during osmotic stress and through Pap1p during exposure to low levels of oxidative stress. However, all three transcription factors are needed for gene expression under conditions of extreme oxidative stress. In addition, a role for Sty1p in the modulation of post-transcriptional activation of trehalase mediated by Pka1p/Sck1p kinases, as well as in the activity of T6P synthase under such stressful conditions has been demonstrated. These results reveal a novel dual action of the Wis1p-Sty1p pathway in the regulation of trehalose metabolism in fission yeast.

**INTRODUCTION**

The response of yeasts to environmental changes involves a dramatic increase in the metabolism of the non-reducing disaccharide trehalose, which functions as a carbohydrate reserve and stress metabolite (Hottiger et al., 1994). Studies *in vitro* and *in vivo* have confirmed the exceptional properties of this sugar in protecting yeast cells under extreme conditions (Crowe et al., 1984; Singer & Lindquist, 1998). In the fission yeast *Schizosaccharomyces pombe*, synthesis of trehalose is a two-step process that includes the intermediate synthesis of trehalose 6-phosphate (T6P) by T6P synthase, encoded by the *tps1*+ gene (Blázquez et al., 1994), and its subsequent dephosphorylation to trehalose by T6P phosphatase, encoded by the *tpp1*+ gene (Franco et al., 2000). On the other hand, hydrolysis of trehalose to glucose is catalysed by the enzyme neutral trehalase, encoded by the *ntp1*+ gene (Soto et al., 1998).

Biosynthesis and mobilization of trehalose are subject to various regulatory controls. The stress-activated protein kinase (SAPK) pathway, a member of the mitogen-activated protein kinase (MAPK) cascades originally described in metazoans, transduces signals to the nucleus, resulting in new patterns of gene expression, and is critical for the sensing and response of *S. pombe* cells to a variety of changes in the external environment (Warbrick & Fantes, 1991; Millar et al., 1995; Shiozaki & Russell, 1995; Kato et al., 1996). The central element of the SAPK cascade in *S. pombe* is the MAPK Sty1p (also known as Spc1p or Phh1p), which is highly homologous to mammalian p38 kinase and becomes activated by a similar range of stresses (Millar et al., 1995; Shiozaki & Russell, 1995; Degols et al., 1996). Different transcription factors function downstream of the Sty1p MAPK cascade, among which Atf1p, Pcr1p and Pap1p have been characterized extensively (Wilkinson et al., 1996; Watanabe & Yamamoto, 1996; Toone et al., 1998). It has been shown previously that the mRNA level of *ntp1*+ and *tps1*+ genes rises when *S. pombe* cells undergo thermal, osmotic or oxidative stresses (Degols et al., 1996; Fernández et al., 1997a, 1998; Cansado et al., 1998; Soto et al., 1998) and that this increase appears to be under the control of the SAPK pathway, since expression of both genes is severely impaired in *S. pombe* strains deleted in the *sty1*+ gene (Degols et al., 1996; Fernández et al., 1998; Soto et al., 1998). To assess further the extent of this regulatory control in the stress-induced expression of *ntp1*+ and *tps1*+ we decided to analyse the role of the transcriptional factors reported to be the downstream targets of the Sty1p MAPK. In the course of these studies we also revealed a role for Sty1p in the...

**Abbreviations:** CRE, cAMP response; MAPK, mitogen-activated protein kinase; Pka1p, protein kinase A; SAPK, stress-activated protein kinase; Sck1p, suppressor of loss of CAMP-dependent protein kinase; T6P, trehalose 6-phosphate.
post-transcriptional modulation of trehalase mediated by Pka1p/Sck1p and in the activity of T6P synthase upon stress.

**METHODS**

**Yeast strains, media and plasmids.** The *S. pombe* strains employed in this study are listed in Table 1. They were routinely grown with shaking at 28 °C in YES medium (Moreno *et al.*, 1991) supplemented with adenine, leucine, histidine or uracil (100 mg l⁻¹) depending on the requirements for each particular strain. Solid media were made by the addition of 2 % (w/v) bacto-agar.

Plasmid pBura4 contains the *ura4*+ gene cloned as two separate *XbaI–BamHI* (950 bp) and *KpnI–Xhol* (850 bp) fragments into pBluescript SKII+. Plasmid pBura4-ntp1+ contains the *ntp1* ORF plus 1.1 kbp of the promoter region cloned as a 3.9 kbp *Xhol–BamHI* fragment into pBura4 and flanked by *ura4*+ fragments.

**Stress treatments, RNA isolation and hybridization.** Yeast cultures grown to an OD₆₀₀ of 0.7–1 at 28 °C were subjected to either saline osmotic stress (0.75 M NaCl) or oxidative stress (0.75 or 5 mM H₂O₂). At different times, the cells from 30 ml culture media were made by the addition of 2 % (w/v) bacto-agar. Total RNA preparations from cold-shocked strains were obtained as described by Moreno *et al.* (1991) and resolved through 1.5% agarose-formaldehyde gels. Northern (RNA) hybridization analyses were performed as described by Soto *et al.* (1998). Probes for *tps1*+ and *ntp1*+ were prepared as reported previously (Fernández *et al.*, 1997a; Cansado *et al.*, 1998). An approximately 900 bp fragment of the *leu1*+ gene was amplified by PCR (Cansado *et al.*, 1998) and used to probe for *leu1*+ mRNA as an internal standard for the amount of RNA loaded in each lane. To establish quantitative conclusions, the level of mRNAs was quantified in a Phosphorimager (Molecular Dynamics) and compared with the internal control (*leu1*+ mRNA).

**Site-directed mutagenesis at the ntp1+ promoter and *S. pombe* transformation.** The mutation at the putative cAMP response (CRE) site (consensus sequence TGACGTAG at position −567) in the *ntp1*+ promoter was created by the overlap extension method with the use of PCR (Higuchi *et al.*, 1988). Two separate amplification reactions were performed with plasmid pBura4-ntp1+ as template with the use of a first pair of primers, PRO52 (5′-TCCGTCGAGATCGTTAGTCAGTC-3′; the *XhoI* site is underlined) and ATFM-3 (5′-TTACTCAATAGGTAGCTACC-3′; the nucleotide substitution is indicated in bold type), and a second pair of primers, NTP3M (5′-ACTGGCAGTTCTCGAGT-3′; the *ClaI* site is underlined) and ATFM-5 (5′-GGTAGACTAGT-CATGTGAGTA-3′; the nucleotide substitution is indicated in bold type). The two PCR products were purified by agarose gel electrophoresis, mixed and subjected again to PCR with primers PRO52 and NTP3M. PRO52 and NTP3M hybridize at positions −1092 to −1075 in the *ntp1*+ promoter and +541 to +561 in the *ntp1*+ ORF, respectively. The resultant 1.7-kbp fragment was digested with *XhoI* and *ClaI* and cloned into pBura4-ntp1+ (CRE), which contains the *ntp1*+ promoter mutated in the putative CRE-binding site plus the complete *ntp1*+ ORF flanked by *ura4*+ sequences.

**RESULTS AND DISCUSSION**

**Role of the Atf1p/Pcr1p heterodimer in the regulation of ntp1+ and tps1+ expression by osmstress**

We performed a quantitative study on the expression of *ntp1*+ and *tps1*+ genes in wild-type (WSP547) and Δsty1

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<th>Table 1. <em>S. pombe</em> strains used in this study</th>
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<td>CA334</td>
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*W. P. Wahls, University of Arkansas, USA; T. Kato, Okayama Cell Switching Project, Japan; T. Toda, London Research Institute, UK; K. Shiozaki, University of California, USA.*
hybridization was performed, employing probes for capillary transferred to nylon membranes. Northern (RNA) or ntp1+ strains grown at 28°C in YES medium and subjected to osmotic (0·75 M NaCl) stress for different times. Total RNA preparations (10 μg) were obtained, resolved through 1·5% agarose-formaldehyde gels and capillary transferred to nylon membranes. Northern (RNA) hybridization was performed, employing probes for tps1+ or ntp1+ and for leu1+ as internal standard. Wild-type cells displayed similar induction kinetics for both ntp1+ and tps1+ genes upon stress (Fig. 1a). The existence of a similar expression pattern for both genes may appear intriguing, since they encode enzymes with opposite biological functions. However, this observation fits other findings suggesting that both synthesis and hydrolysis of trehalose occur in a coordinated way for this sugar to play a protective role.

In S. pombe, a key transcription factor that functions downstream of the Sty1p MAPK cascade is the b-ZIP protein Atf1p (originally reported as Mts1p or Gad7p) (Wahls & Smith, 1994; Takeda et al., 1995). Atf1p forms heterodimers with Pcr1p and associates to, and is phosphorylated by Sty1p following different stresses (Shiozaki & Russell, 1996; Wilkinson et al., 1996). Similar to the effects observed in Δsty1 cells, the loss of Atf1p or Pcr1p produced both a clear delay and a relatively decreased induction of the expression of ntp1+ and tps1+ under osmotic stress that did not increase further even after prolonged periods of time (Fig. 1c, d). The same results were obtained using the double-deleted Δatf1 Δpcr1 strain WSP672 (not shown). These data indicate that Atf1p and Pcr1p transcription factors, likely to be acting as heterodimers, are targets for Sty1p kinase in the regulation of ntp1+ and tps1+ expression in response to osmotic stress. However, the increase in gene expression remaining in Δatf1 and Δpcr1 strains of S. pombe suggests that factors other than Atf1p/ Pcr1p modulate ntp1+ and tps1+ expression during osmotic stress in a Sty1p-independent manner, which highlights the complexity of the stress-induced transcriptional regulation of trehalose metabolism genes.

Pap1p is a member of the AP-1 family of yeast transcription factors which has been reported to control the expression of several genes protecting against oxidative damage (Toone et al., 1998). Experiments similar to those described above, but performed with strain TP108-3c, indicated that pap1+ deficient cells, contrary to atf1+ or pcr1+-disrupted cells, did not substantially differ from wild-type cells in their expression levels of ntp1+ and tps1+ under osmotic stress (not shown).

**Differential involvement of Atf1p and Pap1p in the induction of ntp1+ and tps1+ expression under oxidative stress**

We also analysed the induction of ntp1+ and tps1+ genes in S. pombe during oxidative stress with hydrogen peroxide. Expression of ntp1+ and tps1+ was triggered at low (0·75 mM) and high (5 mM) H2O2 concentrations in wild-type cells, with faster kinetics at low oxidative stimulus (Fig. 2a, b). In the absence of Sty1p MAPK, a smaller but reproducible rise in the expression of ntp1+ and tps1+ was evident at low H2O2 levels, whereas there was no detectable increase at high levels (Fig. 2c, d). These results demonstrate that Sty1p only partially controls ntp1+ and tps1+ expression at low H2O2 concentrations, while it is fully responsible for the induction at a high concentration of oxidative input. Moreover, as compared to wild-type cells, atf1+ disruption (and also pcr1+ deletion; not shown) provoked a decrease...
in gene expression at both high and low H\textsubscript{2}O\textsubscript{2} concentrations (Fig. 2e, f). The fact that Δatf\textsubscript{1} cells still show a significant H\textsubscript{2}O\textsubscript{2}-mediated increase in ntp\textsuperscript{1+} and tps\textsuperscript{1+} expression implies that one or several Sty\textsubscript{1}p-dependent transcription factors are responsible for this effect. One obvious candidate for this additional regulation is Pap\textsubscript{1}p (Toone \textit{et al.}, 1998). Although Pap\textsubscript{1}p is not a substrate directly phosphorylated by Sty\textsubscript{1}p MAPK, the presence of Sty\textsubscript{1}p is critical to allow its translocation from the cytoplasm to the cell nucleus during oxidative stress (Toone \textit{et al.}, 1998). As indicated in Fig. 2(g, h) disruption of the pap\textsubscript{1+} gene resulted in a rather modest increase in ntp\textsuperscript{1+} and tps\textsuperscript{1+} expression, as compared to the wild-type strain, upon treatment with 0.75 or 5 mM H\textsubscript{2}O\textsubscript{2}. A search for AP-1-binding sites (consensus TTAG/CTA/CA) (Toone & Jones, 1999) resulted in the identification of two potential and identical Pap\textsubscript{1}p-binding sites on the complementary strand of the ntp\textsuperscript{1+} promoter at positions −2557 and −2380 (sequence TGAGTAA), and one site in the tps\textsuperscript{1+} promoter at position −2135 (sequence TTAGTAA). Simultaneous deletion of atf\textsubscript{1+} and pap\textsubscript{1+} genes prompted a slight induction at low levels of H\textsubscript{2}O\textsubscript{2}, but no increase in ntp\textsuperscript{1+} expression.

**Fig. 2.** The Atf\textsubscript{1}p and Pap\textsubscript{1}p transcription factors modulate the induction of ntp\textsuperscript{1+} and tps\textsuperscript{1+} expression by H\textsubscript{2}O\textsubscript{2} in a dose-dependent fashion. Strains TK003 (WT), TK107 (Δsty\textsubscript{1}), NT146 (Δatf\textsubscript{1}), TP108-3c (Δpap\textsubscript{1}p) and CA334 (Δatf\textsubscript{1} Δpap\textsubscript{1}p) were grown at 28°C in YES medium and treated with 0.75 or 5 mM H\textsubscript{2}O\textsubscript{2} for the times indicated. Northern blot analyses and symbols are as indicated in the legend to Fig. 1.
and \( tps1^+ \) expression at high \( \text{H}_2\text{O}_2 \) concentrations (Fig. 2i, j). As a whole, these results indicate that the Sty1p-regulated Atf1p/Pcr1p and Pap1p transcription factors are entirely responsible for the increase of \( ntp1^+ \) and \( tps1^+ \) expression at high \( \text{H}_2\text{O}_2 \) concentrations, but not at low concentrations. Quinn et al. (2002) demonstrated that transcription of various genes encoding enzymes involved in \( \text{H}_2\text{O}_2 \) degradation is regulated in a dose-dependent manner, with Pap1p acting mainly at low levels of \( \text{H}_2\text{O}_2 \) (below 1 mM) and Atf1p primarily controlling the transcriptional response to high concentrations. Our results, however, show that both Atf1p and Pap1p are important for \( ntp1^+ \) and \( tps1^+ \) induction at low and high \( \text{H}_2\text{O}_2 \) concentrations (Fig. 2), although other Sty1p-independent factors also appear to be involved at low concentrations of the inducer. Congruent with this, we have observed some nuclear accumulation of a GFP-Pap1p fusion protein after 90 min incubation with 5 mM \( \text{H}_2\text{O}_2 \) (not shown). Considering that the function of \( ntp1^+ \) and \( tps1^+ \) gene products is not directly related to \( \text{H}_2\text{O}_2 \) degradation, it is tempting to speculate that the induction of different sets of genes is distinctly modulated by Atf1p and Pap1p depending on the severity of the oxidative input. Because \( \Delta \text{sty1} \) and other downstream mutants are more sensitive to oxidative stress than wild-type cells it could be argued that decreased expression of \( ntp1^+ \) and \( tps1^+ \) in the mutants might be due to impaired transcription and cell death. However, maintained expression of the \( \text{leu}1^+ \) gene under similar oxidative stress conditions gives compelling evidence that this is not the case. Moreover, functional transcription has been reported in \( \Delta \text{sty1} \) cells at even higher levels of \( \text{H}_2\text{O}_2 \) (Quinn et al., 2002).

Using a threefold increase in the relative expression level as a threshold, Chen et al. (2003) have recently reported Sty1p- and Atf1p-dependent induction of \( ntp1^+ \), but not of \( tps1^+ \), under oxidative conditions. In our hands, however, both genes are expressed above such levels with respect to zero time in a way that is fully dependent on Sty1p at high oxidative input, but only partially dependent on this kinase at low oxidative doses. In addition, the enhanced transcription for \( ntp1^+ \) and \( tps1^+ \) partially relies on Atf1p at high and low inducer concentrations and appears to require Pap1p, particularly under strong oxidative conditions.

**Role of the CRE motif in \( ntp1^+ \) gene expression under stress**

Atf1p is able to bind to CRE-like elements [CRE consensus sequence \( \text{TGACGT(C/A)A} \)] (Takeda et al., 1995; Neely & Hoffman, 2000). In our case, a close inspection of the \( ntp1^+ \) regulatory sequences revealed the existence of a CRE-like element (consensus sequence \( \text{TGACGTAG} \)) at position \(-568\) to \(-561\) in the \( ntp1^+ \) promoter, which was identical to the sequence present in the promoter of the \( fbp1^+ \) gene and identified as a binding site for Atf1p/Pcr1p (Neely & Hoffman, 2000). These data support the existence of an Atf1p/Pcr1p-dependent regulation of \( ntp1^+ \) expression under stress by direct binding to CRE/CRE-like elements. We confirmed this idea by site-directed mutagenesis of the DNA-binding motif in the \( ntp1^+ \) promoter region. The effect of this sequence on \( ntp1^+ \) transcription under stress was studied by constructing strain MVP-12, in which \( ntp1^+ \) expression is regulated by an endogenous 1·1 kbp promoter carrying a base change (G to C) within the core ACGT sequence of the CRE-like element (see Methods). As shown in Fig. 3, the control strain MVP-10, which contains a wild-type non-mutated 1·1 kbp fragment of the \( ntp1^+ \) promoter, displayed a pattern of \( ntp1^+ \) expression under salt and oxidative stress similar to wild-type strains (see Figs 1 and 2), indicating that the main upstream activating sequences involved in the stress-induced expression of \( ntp1^+ \) are located in this region. A single G to C change at the CRE-like element (strain MVP-12) caused an overall decrease in \( ntp1^+ \) expression upon osmotic or oxidative stress.

**Fig. 3.** The CRE-like element present in the \( ntp1^+ \) promoter is required for stress-induced gene expression. The control strain MVP-10 and its isogenic counterpart MVP-12, carrying a G to C change within the ACGT core sequence of the CRE-like element, were subjected to osmotic or oxidative stress for the indicated times. Total RNA was extracted and analysed by Northern blotting using a \( ^{32}\text{P} \)-labelled \( ntp1^+ \) probe. The transcript levels from each strain were normalized to \( \text{leu}1^+ \) levels and the relative fold induction compared in each case.
stress quite similar to that found in \textit{atf1}^+ -disrupted cells under the same conditions (Figs 1 and 2). Hence, the role for Atf1p in the regulation of \textit{ntp1}^+ expression under stress appears to operate through interaction with the CRE-like sequence located in the promoter. In addition to binding to CRE-like sequences, Atf1p is also specifically phosphorylated by Sty1p MAPK under salt and oxidative stresses (Wilkinson \textit{et al}., 1996). Therefore, it is likely that the enhanced gene expression during salt and oxidative stresses occurs by direct binding of Atf1p in a phosphorylated form to a CRE-like motif at the \textit{ntp1}^+ promoter. In addition, we also detected a putative CRE element (sequence AGACGTA) in the \textit{tps1}^+ promoter at position −180. Although not proved, it is possible that the Atf1p-mediated expression of \textit{tps1}^+ may be regulated in a similar way to \textit{ntp1}^+. This suggestion correlates with the observation that both genes are expressed in parallel under different stresses.

**Stress-induced neutral trehalase activation and trehalose pool in \textit{S. pombe} strains devoid of \textit{Sty1p}-regulated transcription factors**

The product of the \textit{ntp1}^+ gene, neutral trehalase (Ntp1p), is activated by phosphorylation under stress conditions (Carrillo \textit{et al}., 1994; Fernández \textit{et al}., 1997a, b, 1998). We reported previously that cells disrupted in the \textit{Sty1p} MAPK show a marked reduction in trehalase activity during osmostress or oxidative treatment and suggested a limited \textit{de novo} synthesis of the enzyme protein under these conditions (Fernández \textit{et al}., 1997b, 1998). Because Atf1p/Pcr1p and Pap1p transcription factors are the main downstream elements involved in \textit{ntp1}^+ expression under osmotic and oxidative stresses (Figs 1 and 2), it should be expected that the absence of these factors would markedly affect Ntp1p activation upon these stress treatments. To ascertain this assumption, \textit{S. pombe} wild-type strain and \textit{Δsty1}, \textit{Δatf1}, \textit{Δpap1} or \textit{Δatf1 Δpap1} mutant strains were subjected to osmotic or oxidative stress, and neutral trehalase activity was measured at different periods of treatment. Surprisingly, \textit{Δatf1}, \textit{Δpap1} and \textit{Δatf1 Δpap1} cells displayed a wild-type pattern of neutral trehalase activation whereas only \textit{Δsty1} cells were unable to increase neutral trehalase activity in response to high osmolarity (Fig. 4a) or oxidative stress (Fig. 4b). Since activation of trehalase by osmostress or oxidative stress relies on the function of Pka1p and Sck1p protein kinases (Fernández \textit{et al}., 1997b, 1998), the above results reflect the fact that the relatively low level of trehalase activity in osmotic- and oxidative-stressed \textit{Δsty1} cells is due to a post-translational event rather than to decreased \textit{ntp1}^+ expression. Therefore, Sty1p appears to be needed not only for proper \textit{ntp1}^+ induction in response to these stresses (Figs 1 and 2), but also for modulating the function of Pka1p and Sck1p kinases in the stress-induced activation of neutral trehalase. In this context, loss of \textit{sty1}^+ function appears epistatic over Pka1p and Sck1p.

A general feature shown by yeast cells is a net increase in the trehalose pool upon stress (Hottiger \textit{et al}., 1994). In line with the above results we also analysed the trehalose content in wild-type, \textit{Δsty1}, \textit{Δatf1}, \textit{Δpap1} and \textit{Δatf1 Δpap1} cells after osmotic or oxidative treatment as a measure of \textit{in vivo} T6P synthase activity. As indicated in Fig. 5, the level of trehalose in \textit{Δsty1} cells was greatly reduced as compared to that shown in wild-type or in cells disrupted in the transcription factors. This supports that control by Sty1p also affects trehalose synthesis in a way that is similarly independent of Atf1p and Pap1p. Notably, \textit{Δwis1} cells, which contain undisrupted Sty1p, show the same phenotype as \textit{Δsty1} cells with respect to decreased trehalase activation and trehalose content upon stress (Fernández \textit{et al}., 1997b, 1998), demonstrating that Sty1p phosphorylation, rather than the presence of the kinase enzyme protein, is needed for normal trehalase activation and trehalose accumulation.
These results raise the question of how Sty1p, which shifts to the nucleus upon activation by stress (Wilkinson & Millar, 1998), exerts its action on the cytoplasmic enzymes trehalase and T6P synthase. This point remains unresolved but the nuclear localization of the SAPK argues against a direct inhibition of the Atf1p/Pcr1p heterodimer by Pka. The SAPK and Pka pathways are known to antagonistically regulate different biological processes in Schizosaccharomyces pombe. The SAPK and Pka pathways are known to antagonistically regulate different biological processes in S. pombe (Maeda et al., 1994; Caspari, T., 1997; Fernández et al., 1997a). At the transcriptional level, for example, both pathways regulate fbp1 expression by favouring (SAPK) or inhibiting (Pka) the binding of the Atf1p/Pcr1p heterodimer to the fbp1 promoter (Neely & Hoffman, 2000). It is also known that Sty1p affects pka1 expression upon stress (Chen et al., 2003), suggesting that the inability for normal trehalase activation in cells lacking sty1 might result in part from decreased transcription of pka1. However, in contrast to trehalase activity, there is no evidence for regulation of T6P synthase activity by phosphorylation, leading to the idea that the effect of Sty1p on the synthase enzyme occurs by a different pathway independent of Pka1p/Sck1p protein kinases. Although we have yet to define precisely how Sty1p influences the activity of these enzymes, our findings reveal a new regulatory function for this kinase in the metabolism of trehalose during stress.

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