Activity of the circadian transcription factor White Collar Complex is modulated by phosphorylation of SP-motifs

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ABSTRACT

Posttranslational modifications, particularly phosphorylation, regulate activity, stability and localization of proteins in circadian clocks, thereby contributing to a stable oscillation with a period of approximately 24 h. The White Collar Complex (WCC) is the central transcription factor of the circadian clock of Neurospora crassa. Its activity is regulated in a circadian manner by rhythmic phosphorylation, mediated by the clock protein Frequency (FRQ). Here we present purification of TAP-tagged WCC and identification of novel phosphorylation sites of WC-1 and WC-2, all of which appear to be proline directed. Exchange of a single WC-2 serine residue (S433) to alanine or aspartate affects WCC-dependent transcription and circadian period, suggesting an important role of WC-2 S433 phosphorylation for WCC activity and circadian timing.

1. Introduction

Circadian clocks are cell-based oscillators, which enable organisms to anticipate daytime specific changes of environmental parameters such as light, temperature and humidity. The molecular clocks of eukaryotes are constituted by interconnected transcriptional and translational feedback loops, which directly or indirectly drive the cyclic expression of a large number of genes, thereby generating ∼24 h ("circadian") oscillations of physiological and behavioral functions [1–3]. Several delay mechanisms that are mostly based on posttranslational protein modifications contribute to and are essential for a stable and robust oscillation with a ∼24 h period [4–6]. However, the molecular basis of circadian timekeeping remains to be elucidated.

The core components of the Neurospora clock are the PAS-domain transcription factors WC-1 and WC-2, which form a heteromeric complex (White Collar Complex, WCC) that drives the expression of the negative clock element Frequency (FRQ) [7,8]. WCC binds to the frq promoter in a light dependent and in a circadian manner [9,10]. FRQ represses its own transcription by mediating inactivation of WCC by casein kinase 1α-dependent phosphorylation [11]. In consequence the overall phosphorylation state of WC-2 oscillates with a circadian period, WC-2 is apparently phosphorylated on more than eight sites [12]. FRQ also acts as a positive element of the clock, supporting accumulation of the WCC [13–15]. Negative and positive feedback by FRQ underly the same molecular mechanism: Hypophosphorylated WCC is active but unstable. FRQ-dependent phosphorylation of WCC results in inactivation and stabilization, leading to accumulation of newly synthesized WCC [16].

Although the regulation of WCC activity by FRQ-dependent phosphorylation is well characterized [12], only a few phosphorylation sites of WC-1 have been identified [17,18] and no WC-2 phosphorylation sites have been identified to date.

We have established a new approach for purification of the WCC by tandem affinity chromatography. We confirmed recently identified phosphorylation sites of WC-1 by mass spectrometry of purified WCC. Furthermore we mapped additional phosphorylation sites of WC-1 and identified the first phosphorylation site of WC-2 (S433). All sites appear to be targets for proline-directed phosphorylation. We have characterized S433 and propose a role of S433 phosphorylation in the regulation of WCC transcriptional activity and control of circadian period length.

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2. Materials and methods

2.1. Strains and growth conditions

All strains carried the ras<sup>bd</sup> mutation [19]. Plasmid constructs were inserted into the his-3 locus and strains were grown as described previously [20]. The control wt strain is Δwc-2, bd transformed with wild-type wc-2 gene with its endogenous 2.2 kb promoter [20]. Site-directed mutagenesis of mutant wc-2 was done using Stratagene QuikChange® Site-Directed Mutagenesis Kit according to the manufacturer’s protocol. The mutagenesis primers are listed below:

wc-2 433S-A fwd: 5′-cat ggt atc aca acg aat gcc cgg acg ctc att aag-3′
wc-2 433S-A rev: 5′-ctt aat gag cgt cgg gcc ggc att acc cgt tgt gat acc atg-3′
wc-2 433S-D fwd: 5′-cat ggt atc aca acg aat gcc gat cgg acg ctc att aag-3′
wc-2 433S-D rev: 5′-ctt aat gag cgt cgg gcc att acc cgt tgt gat acc atg-3′

2.2. Construction of qa-tap-wc-2

A Tap-tag was amplified with the primers listed below and performed a one-step enrichment of TAP-WC-2 on IgG agarose [26].

qa2 433S-A fwd: 5′-agg cat ggg gat gcc agg cct tgt gac ac-3′
qa2 433S-A rev: 5′-tcc cat ccc cat ggc aag tgc ccc gga-3′

2.3. Tandem-affinity purification of White Collar Complex

TAP purification method was optimized for Neurospora crassa based on original protocol [21]. A qa2-tap-wc2 strain was grown in light for 3 days in standard medium and transferred to induction medium [14]. Induction was done with 0.3% quinic acid for 12 h. The frozen mycelia was ground and cells were lysed by vortexing in TAP buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF, 1 μg/ml Leupeptin, 1 μg/ml Pepstatin A). A pre-clearing centrifugation step was done for 20 min at 10,000× g. Supernatants were combined and ultracentrifuged (38 k rpm 1 h, Sorvall WX ultracentrifuge, rotor T647.5). Cleared protein extracts (~2.5 g) were incubated with 500 μl IgG beads (GE Healthcare) for 4 h at 4 °C. IgG beads were transferred to 1 ml Mobicol columns (Mobitec) and washed with 40 ml TAP buffer with protease inhibitors. One hundred units of tobacco etch virus (TEV) protease (Invitrogen) in 500 μl TEV cleavage buffer (TAP buffer with 1 mM DTT) were added to the column and incubated for 4 h at 16 °C. Finally 2 mM CaCl<sub>2</sub> was added to the IgG elution and incubated with 350 μl CaM beads (GE Healthcare) for 2 h at 4 °C. CaM beads were transferred to 1 ml Mobicol columns and washed with 40 ml CaM buffer (TAP buffer with 2 mM CaCl<sub>2</sub>). Elution was done 3 x 10 min with 400 μl CaM elution buffer (TAP buffer with 4 mM EGTA) at RT. The protein eluate was TCA-DOC precipitated and the pellet was dissolved in 20 μl 2 × LDS sample buffer (Invitrogen) with 50 mM DTT and boiled at 72 °C for 10 min.

2.4. Mass spectrometry

SDS–PAGE for mass spectrometry was done by using 4–12% precast bis–tris gels with MOPS running buffer (Invitrogen) at 200 V for 1 h. The gel was stained with Roti®-Blue colloidal coomassie from (Roth) according to the manufacturer’s protocol. The mass spectrometry for identification of the proteins and the phosphorylation sites were performed as described [22,23].

2.5. Protein analysis

Protein was extracted from mycelia by previously described methods [24]. Total protein (200 μg) per lane was subjected to SDS–PAGE on 12% gels. Western blots were decorated with monoclonal antibodies against FRQ or affinity-purified rabbit antibodies against WC-1 and WC-2. Western blots were performed using enhanced chemiluminescence (ECL). To control uniform loading of the gels, nitrocellulose filters were stained with Ponceau S.

2.6. RNA analysis

RNA was prepared using peqGOLD TriFast<sup>™</sup> (peqLab, Erlangen, Germany), reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Transcript levels were analyzed by quantitative real time PCR as described before [25].

2.7. Race tube analysis

Race tubes were inoculated, germinated in LL for ~24 h and transferred to DD at 25 °C. The growth front was marked under red light every 24 h. After 5–6 days race tubes were scanned and the conidial densities were quantified using the Chrono program [26].

3. Results

3.1. Purification of TAP-tagged WC-2

In the recent years efforts were made to obtain pure WCC from Neurospora cultures, e.g. by expression and purification of myc-tagged WC-2 [18]. Due to the low expression level of the circadian transcription factor however, it is difficult to gain large amounts of pure WCC. We constructed a version of WC-2 with an N-terminal tandem affinity tag (TAP-WC-2), consisting of calmodulin binding peptide and protein A, which can be separated by a TEV-cleavage site (Fig. 1A). The tagged protein was expressed in Neurospora under control of the inducible qa-2 promoter and supported free-running rhythms, demonstrating that it was active in the circadian clock (Fig. 1A). Short WC-2 (s-WC-2), an N-terminally truncated version of WC-2 which is expressed under control of a promoter located within the ORF [20], was expressed even in the absence of QA, but does not carry a TAP-tag. After tandem-affinity purification (CaM), two proteins of the expected sizes of WC-1 and WC-2 were highly enriched, as estimated from coomassie- and silver-stained SDS-gels (Fig. 1B and C). The identity of the proteins was confirmed by mass spectrometry. Two additional bands present in substantial amount were identified as a heat-shock protein (HSP70) and eukaryotic elongation factor 1α (eEF1α). However, HSP-70 and eEF1α were also detected in a control purification (Fig. 1D and data not shown), suggesting that they are unspecific contaminants. The data suggest that the WCC is composed of WC-1 and WC-2 subunits, which, based on coomassie staining, appear to be present approximately at 1:1 ratio. No further protein is stable associated with the WCC in stoichiometric amounts.

Components that are only loosely associated with WCC might get lost in the course of the purification procedure. We therefore performed a one-step enrichment of TAP-WC-2 on IgG agarose
beads and eluted with TEV-protease. Despite a considerably high background WC-1 and WC-2 were highly enriched in the eluate fraction. However, no other protein copurified with WCC above background level (Fig. 1D, left).
To detect potential interaction partners that are only loosely associated with WCC we used in vivo cross-linking with formaldehyde (FA), which forms methylene bridges between ε-amino groups of lysines and the amide group of the peptide backbone. A physical interaction of the WCC and FRQ has been extensively discussed in the last decade [12,27]. Recently, our group could show that a stable assembly of WCC and FRQ is not required for inactivation of the transcription factor [12]. Nevertheless, inactivation of WCC by FRQ-dependent phosphorylation implies a certain degree of interaction. To trap loosely or transiently attached FRQ we cross-linked TAP-WC-2 expressing cultures in vivo with 0.1% FA. Since we could not detect reasonable amounts of specifically purified proteins other than WC-1 and WC-2 by TAP (Fig. 1D, right), we analyzed the eluated fractions by Western blotting, using a monoclonal antibody against FRQ. Although not visible by silver staining, small but detectable quantities of FRQ could be detected after in vivo cross-linking with 0.1% FA (Fig. 1E), suggesting a weak but specific interaction of FRQ and WCC.

In summary the data indicate that WCC can be enriched to high purity by TAP, but other interaction partners are not traceable in significant amounts by using this method.

3.2. Determination of phosphorylation sites by mass spectrometry

The transcriptional activity of WCC is determined by its phosphorylation state. Five light-independent phosphorylation sites in WC-1 have been identified recently [18]. We have shown that WC-2 is phosphorylated at up to eight sites and that the overall phosphorylation of WC-2 is oscillating in a circadian manner with hyperphosphorylation correlating with low activity of WCC [12]. However, phosphorylation sites of WC-2 have not been mapped to date.

To identify new phosphorylation sites in TAP-tagged WCC was purified and protein bands corresponding to WC-1 and WC-2 were analyzed by mass spectrometry. The sequence coverage was 46% for WC-1 and 36% for WC-2. We confirmed three of the five known WC-1 phosphorylation sites. In addition, we identified four new sites in WC-1 and one in WC-2 (Table 1 and Fig. 1F). Interestingly, all identified sites possess a SP motif. This suggests that these sites might be targets of proline-directed kinases.

Table 1

| Protein | Phosphorylated residue | Peptide |
|---------|------------------------|---------|
| WC1     | S-990, S-998 or S-995 | EFGNDSpSPTTATK |
|         | S-1015                 | S-1005 |
| WC2     | S-433                  | SHGiTTGNaPSTLIK |

Fig. 1 (continued)
3.3. WC-2. S433-phosphorylation regulates WCC activity

To investigate the role of phosphorylation of serine 433 in WC-2, we constructed mutated forms of the \textit{wc-2} gene under control of the endogenous promoter and expressed them in a \textit{Dwc-2} strain. In one case S433 was exchanged to an alanine residue to prevent phosphorylation (S433A), in the other case it was exchanged to aspartate, which is commonly used to mimic constitutive phosphorylation of serine residues (S433D). The corresponding strains are referred to as S433A and S433D. First, we investigated the expression levels of the WCC subunits WC-2 and WC-1 in the mutant strains.

Phosphorylation regularly targets proteins for degradation. However, WC-2 S433A levels were slightly lower than WC-2 levels in the \textit{wt} control strain (\textit{Dwc-2} transformed with wild-type \textit{wc-2}) at least in LL, although stability of WC-2 was not affected (Fig. 2A and data not shown). On the contrary, expression levels of WC-2 S433D were somewhat increased in constant light (LL) as well as constant darkness (DD) (Fig. 2A). Despite higher WC-2 levels in S433D, protein stability is not affected (data not shown). The levels of WC-2 in the mutant strains were reflected by the amount of \textit{wc-2} mRNA (Fig. 2C). The data suggest that \textit{wc-2} transcription is reduced in S433A and enhanced in the S433D strain. WC-1 protein and \textit{wc-1} mRNA levels were not affected in S433A and S433D (Fig. 2B and D). These findings indicate that the levels of WCC are similar in wild-type and in the mutant strains. Hence, the levels of free WC-2 are elevated in S433D.

WCC transcription is indirectly inhibited by WCC [20]. Our data suggest that WCC is more active in S433A and less active in S433D. To investigate the WCC-activities we analyzed expression levels of \textit{frq}, which is transcribed under control of WCC. Interestingly, FRQ protein and mRNA levels were increased in S433A and decreased in S433D (Fig. 3A and B). Corresponding effects were measured by analysis of \textit{vvd} expression, a gene that is also under direct control of WCC (data not shown). These observations indicate an elevated activity of WCC in S433A and a reduced activity in S433D.

The reported data show steady-state expression of WCC-dependent genes in LL and DD. To analyze light induced transcription, cultures of \textit{wt}, S433A and S433D were grown in DD for 24 h and transferred into high light (120 \textit{l E}). Transcript levels of \textit{vvd} were analyzed by quantitative RT-PCR. Light induction and adaptation of \textit{vvd} RNA in \textit{wt} and S433D displayed similar kinetics. However, maximal levels of \textit{vvd} RNA are slightly lower in S433D than in \textit{wt} (Fig. 3C). The data suggest that the majority of WC-2 is phosphorylated at S433 in \textit{wt}. In S433A \textit{vvd} mRNA levels increased faster than in \textit{wt} and S433D supporting the notion that the dephosphorylated version is more active (Fig. 3C). Light adaptation was also faster in S433A presumably because VVD protein was synthesized more rapidly (Fig. 3C). The data confirm our assumption that WCC activity is reduced by phosphorylation and enhanced by dephosphorylation of S433.

Taken together, our data suggest that the obligatory unphosphorylated form S433A is more active than the wild-type and the variant mimicking constitutive phosphorylation is less active.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{wc-1 and wc-2 RNA and protein levels in S433A and S433D. Cultures were grown for 2 days in constant light (LL) or transferred to constant darkness (DD) for 24 h after 1 day in LL. (A) Upper panel: Western blot showing WC-2 levels in S433A and S433D compared to wt. Analysis of two clones of each mutant was performed with similar results. Lower panel: Quantification of WC-2 levels normalized to tubulin from blots as shown above (means ± S.E.M., n = 4). (B) Blots from (A) were decorated with WC-1 antiserum and quantified (means ± S.E.M., n = 4). (C) Real-time PCR analysis of wc-2 RNA levels of S433A, S433D and wt. Graph represents means ± S.E.M. of three independent experiments. (D) Real-time PCR analysis of wc-1 RNA as shown in C.}
\end{figure}
3.4. Phosphorylation of WC-2 S433 modulates circadian period length

Elevated levels of FRQ generally result in a lengthening of the circadian period by keeping the WCC inactive for a longer time [25]. Hence, one would expect a shorter period when WCC activity is increased. To test the influence of S433 phosphorylation on period length we performed race tube analysis, a common assay to investigate the period length of the conidiation rhythm of Neurospora. The wild-type strain displays a conidiation rhythm with an average period of 22.52 ± 0.12 h (Fig. 4). The period length of S433A was slightly shortened by almost half an hour (22.04 ± 0.13 h; Fig. 4). In contrast, the period of S433D was lengthened by 1.5 h (23.97 ± 0.15 h; Fig. 4). In summary, S433A displayed a 2 h shorter rhythm than S433D, supporting the observation that WCC is more active in S433A than in S433D.

4. Discussion

The activity of WCC, the central transcription factor in the circadian clock of N. crassa, is regulated in a negative feedback loop by phosphorylation supported by FRQ [12]. In this study we affinity-purified WCC and identified by mass spectrometry four new and three known phosphorylation sites of WC-1. We also identified the first phosphorylation site in WC-2. Most probably due to technical reasons, only one of at least eight phosphorylation sites of WC-2 was identified by analysis of trypsin fragments of WC-2. Many of the predicted trypsin fragments are too large for analysis by mass spectrometry and thus, the sequence coverage was only 36%. Hence, different proteases could be used in future approaches to identify additional phosphorylation sites.

We functionally characterized phosphorylation of serine 433 (S433) of WC-2. Analysis of strains harboring WC-2 variants with exchanges of S to A preventing phosphorylation and S to D mimicking constitutive phosphorylation indicated that S433 plays a role in regulation of WCC activity but does not affect WCC turnover. The activity of WCC was elevated in the S433A strain, as evident by increased frq RNA levels in steady state and faster light induction of vvd RNA. Vice versa, WCC was less active in the S433D strain, i.e. frq transcript levels were reduced. Light adaptation was advanced in S433A, probably due to more rapid accumulation of the blue-light...
Recently we have shown that this inhibition is indirect via an unidentified repressor of wc-2 transcription, which is expressed under control of WCC [20]. The level of wc-2 mRNA was elevated in s433d and lowered in s433a supporting the notion that the activity of WCC is reduced by phosphorylation of S433.

Interestingly, the novel phosphorylation sites identified in this study are located directly upstream of a prolyl residue (Table 1), suggesting phosphorylation of these sites by proline-directed serine/threonine kinases such as MAPK-activated protein kinase (MAPK) and cyclin-dependent kinase (CDK) [30–32].

It has been shown that PKA supports directly or indirectly phosphorylation of S990 and S995 of WC-1 [17]. Phosphorylation of these sites is independent of FRQ and can be extended in FRQ-dependent fashion presumably by casein kinase 1 (CK1) and CK2 [11,17]. The sequence context around residues S990 (KSSNP) and S995 (SHSSP) indicates that S990 might be a direct target of PKA, while S995 is not a typical PKA site (R-X-S/ T or R-R/K-X-S/ T) [33]. Both sites are part of SP motifs predicted to be phosphorylated by CDK or MAPK. The newly identified WC-1 phosphorylation sites as well as WC-2 S433 are not in good context for phosphorylation by CK1 and CK2.

S433 of WC-2 is part of a SP motif. Therefore, phosphorylation of S433 might be independent of precedent phosphorylation events, similar to phosphorylation of S990 and S995 of WC-1.

Phosphorylated SP-motifs are recognized by the peptidylprolyl cis-trans isomerase Pin-1. Hence, phosphorylation of such sites can result in Pin-1-dependent conformational changes [31]. It will be interesting to see whether Pin-1 has role in regulation of WCC activity.

Based on our functional analysis of the s433a and s433d alleles, phosphorylation of S433 has a small but distinct impact on WCC activity and circadian rhythmicity. The activity of WCC oscillates in circadian manner and correlates with rhythmic phosphorylation of the WC-2 subunit [12]. At any particular time point of a circadian period WC-2 is heterogeneously phosphorylated at up to eight or more sites. WC-1 is also phosphorylated at multiple sites, though little is known about circadian oscillation of its phosphorylation state. The overall phosphorylation of WC-2 and WC-1 appears to be independent of phosphorylation of WC-2 at S433, suggesting S433 is not a critical priming phosphorylation site. It seems therefore likely that WCC activity is not regulated by a single on/off switch. Rather, phosphorylation of individual sites may only partially and slightly reduce WCC activity. Phosphorylation at multiple residues may synergistically contribute to inactivation of the transcription factor similar to regulation of the hetero-dimeric transcription factor Pho2/4 in Saccharomyces cerevisiae. The activity of Pho2/4 is negatively regulated by proline-directed phosphorylation of the Pho4 subunit at five sites catalyzed by the cyclin-dependent kinase Pho85 in complex with the cyclin Pho80 [34–36]. Phosphorylation at distinct sites affects DNA binding, nucleo-cytoplasmic shuttling and hetero-dimerization, and thus each site contributes independently to partial inactivation of the transcription factor by different means. This facilitates fine-tuning of transcription factor activity in response to external phosphate concentration. Corresponding regulation of the WCC could facilitate a smooth activity rhythm on a circadian time scale. S433 of WC-2 is close to the nuclear localization signal (NLS) [16] and Zn-finger domain of WC-2 suggesting that phosphorylation of S433 may regulate nuclear localization or DNA binding of the transcription factor. Phosphorylation of WC-2 at other sites may affect the activity of WCC by different means.

Acknowledgements

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