Yeast Carboxyl-terminal Domain Kinase I Positively and Negatively Regulates RNA Polymerase II Carboxyl-terminal Domain Phosphorylation

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Monoclonal antibodies that recognize specific carboxyl-terminal domain (CTD) phosphoepitopes were used to examine CTD phosphorylation in yeast cells lacking carboxyl-terminal domain kinase I (CTDK-I). We show that deletion of the kinase subunit CTK1 results in an increase in phosphorylation of serine in position 5 (Ser5) of the CTD repeat (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7) during logarithmic growth. This result indicates that CTDK-I negatively regulates CTD Ser5 phosphorylation. We also show that CTK1 deletion (ctk1Δ) eliminates the transient increase in CTD serine 2 (Ser2) phosphorylation observed during the diauxic shift. This result suggests that CTDK-I may play a direct role in phosphorylating CTD Ser2 in response to nutrient depletion. Northern blot analysis was used to show that genes normally induced during the diauxic shift are not properly induced in a ctk1Δ strain. Glycogen synthase (GSY2) and cytosolic catalase (CTT1) mRNA levels increase about 10-fold in wild-type cells, but this increase is not observed in ctk1Δ cells suggesting that increased message levels may require Ser2 phosphorylation. Heat shock also induces Ser2 phosphorylation, but we show here that this change in CTD modification and an accompanying induction of heat shock gene expression is independent of CTDK-I. The observation that SSA3/SSA4 expression is increased in ctk1Δ cells grown at normal temperature suggests a possible role for CTDK-I in transcription repression. We discuss several possible positive and negative roles for CTDK-I in regulating CTD phosphorylation and gene expression.

The carboxyl-terminal domain (CTD)3 of the RNA polymerase II largest subunit is comprised of tandem repeats of the consensus sequences Tyr-Ser-Pro-Thr-Ser-Pro-Ser (1–4). The CTD has been implicated in many stages of the transcription cycle including transcription initiation, elongation, and pre-mRNA processing (5–10), but its precise mode of action has not been determined.

The CTD is heavily phosphorylated in vivo (11), and this is an essential modification for vegetative growth in yeast (12). Phosphorylation of the CTD is temporally linked to the transition between initiation and elongation in vitro (13–15) leading to a model in which pol II with an unphosphorylated CTD (pol II) participates in forming the initiation complex, and pol II with a phosphorylated CTD (pol II0) is engaged in transcript elongation (16).

Among the best characterized CTD kinases are those present in pol II preinitiation and/or elongation complexes. The TFIIH-associated Cdk7/cyclin H (Kin28/Ccl1 in yeast) (17–22) and Cdk8/cyclin C (Srb11/Srb10 in yeast) (23–25) can both phosphorylate the CTD in vitro and are well positioned for participation in promoter clearance. Much attention has also been paid to P-TEFb, an elongation factor (26, 27) that contains an in vivo CTD kinase activity (28) comprised of Cdk9/cyclin T (29–32). Consistent with its requirement for productive elongation (28), P-TEFb remains with pol II during elongation (33).

Yeast CTDK-I is a Cdk-cyclin kinase that is closely related to P-TEFb, although whether these kinases perform the same function in vivo has not been determined. CTDK-I was isolated as a complex that specifically phosphorylates the CTD in vitro (34). CTDK-I is comprised of three subunits encoded by CTK1, CTK2, and CTK3 (35). CTK1 encodes a kinase catalytic subunit closely related to the P-TEFb Cdk9 subunit (29). The Ctk2 protein resembles cyclin T, whereas Ctk3 shows no homology to known proteins (35). Like P-TEFb, CTDK-I has been shown to stimulate pol II elongation in vitro (36). This function is not essential, however, as deletion of any single or all of the genes encoding CTDK-I is not lethal in yeast (35, 37). CTDK-I-deficient strains grow more slowly than wild type at normal temperatures and are unable to grow at low temperature (35, 37) indicating that some cellular processes are impaired. Examining the phosphorylation state of the CTD in CTK1-deleted (ctk1Δ) cells initially indicated that the CTD was not phosphorylated in the normal fashion (37). In particular, the abundance of a slower mobility form of the largest subunit (Rpb1p) was reduced suggesting that the CTD was under-phosphorylated. Reactivity with anti-phospho-CTD antiserum remained, however, suggesting the existence of multiple CTD kinases in vivo. These studies did not address the identity of the sites phosphorylated by CTDK-I nor the sites that remain phosphorylated in its absence.

Standard approaches to mapping in vivo CTD phosphorylation sites are impractical due to the repetitive nature of the amino acid sequence. Less direct in vitro approaches have yielded important information about potential phosphorylation sites. For example, we showed that both serine 2 (Ser2) and serine 5 (Ser5) of the consensus heptapeptide can be phosphorylated in vitro by Cdc2 kinase (38), and mutation of these sites to alanine in the yeast CTD is lethal (12). Together these
results suggest that these serine residues act as phosphoacceptors for one or more CTD kinases in vivo.

More recently, we mapped critical elements of the CTD phosphoepitopes recognized by a set of monoclonal antibodies (39). By using in vitro phosphorylated CTD fusion proteins, we showed that mAb H14 specifically binds CTDs phosphorylated at Ser5, whereas mAb H5 specifically binds CTD fusion proteins phosphorylated at Ser2. These antibodies bind in vivo phosphorylated CTD indicating that both Ser2 and Ser5 are phosphorylated in vivo. We further showed that Ser2 and Ser5 phosphorylation is independently regulated during yeast growth (39).

We now extend the characterization of CTD phosphorylation by showing that deletion of CTK1 results in a profound increase in phosphorylation of Ser5. In addition, the increase in Ser2 phosphorylation previously observed during the diauxic shift is not observed in ctk1Δ cells. Taken together, these results suggest that CTDK-I participates in both positive and negative regulation of CTD phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The strains used in this study are as follows: ADH6–1a (MATa ctk1Δ::HIS3 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-321); ADH6–1b (MATa CTK1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-321); MCY3664 (MATa ctk1Δ::HIS3 ura3-52 trp1-Δ63 his3-Δ200 leu2-321); MCY3661 (MATa CTK1 ura3-52 trp1-Δ63 his3-Δ200 leu2-321) (35, 40). Media and growth conditions for yeast are as described earlier (39).

**Heat Shock**—Yeast strains ADH6–1a and ADH6–1b were grown in YPD in a water bath at 30 °C to an *A_{sof} of 1.0. The culture (75 ml in a 500-ml flask) was then shifted to a 42 °C water bath for 30 min. Control cells were maintained at 30 °C for the same time. The heat shock was terminated by diluting the culture with ice-cold water, and cells were harvested by centrifugation and extracts prepared as described earlier (39).

**Antibodies**—mAbs 8WG16, H5, and H14 have been described earlier (39, 41). Anti-JC20 antibodies were raised in rabbit against a nine amino acid peptide (MVGGQYQYSSA) corresponding to the amino terminus of the largest subunit (Rpb1p) of yeast pol II. These antibodies were subsequently affinity purified on a peptide column (Affi-Gel, Bio-Rad) and were used at a dilution of 1:200 for Western blot analysis. Anti-Ssa1p (42) and anti-Ssa3p/Ssa4p (43) antibodies were kind gifts from Dr. David Meyer (University of California, Los Angeles) and Dr. Elizabeth Craig (University of Wisconsin Medical School), respectively. These antibodies were used at a dilution of 1:5000. Anti-Ssa3p,4p detects both Ssa3p and Ssa4p, and anti-Ssa1p recognizes all four Ssa proteins (42, 43). The mAb anti-Nab3p antibody (44) was kind gift from Dr. Maurice Swanson (University of Florida Medical School).

**RESULTS**

**Changes in CTD Phosphorylation in CTK1 Null Strains**—Earlier studies showed that deletion of CTK1 results in an apparent decrease in CTD phosphorylation as determined by an increase in electrophoretic mobility of the largest subunit and a decreased reactivity with polyclonal antibodies raised against in vitro phosphorylated CTD (37). However, this study did not address which of the known phosphoepitopes were affected. We have used a set of monoclonal antibodies that recognize different CTD phosphoepitopes to characterize the CTD phosphorylation patterns in ctk1Δ cells relative to wild-type cells. The antibodies employed include the following: mAb 8WG16 which recognizes unphosphorylated Ser2; mAb H5 which recognizes phosphorylated Ser3; and mAb H14 which recognizes phosphorylated Ser5 (39). These antibodies were used to examine the phosphorylation state of the CTD in total cell extracts prepared from growing yeast cells.

The results presented in Fig. 1A clearly indicate an increase in the mAb H14-reactive epitope in the ctk1Δ strain suggesting an increase in Ser2 phosphorylation. In multiple experiments we have consistently observed a 3–5-fold increase in H14 reactivity when equal amount of protein is loaded. This increase in H14 reactivity is not due to an increase in the amount of pol II as determined from the reactivity of other pol II-specific antibodies. We also see a similar increase in mAb H14 reactivity in two other independently derived ctk1Δ strains MCY3664 (40) and YJC1160 indicating that the increase in H14 reactivity is not specific to the ADH6 background (35).

A consistent with an increase in CTD phosphorylation, we see a decrease in the mobility of Rpb1p detected with mAb 8WG16. This antibody recognizes the Ser2 site in unphosphorylated repeats. It thus refers both with the hypo-phosphorylated IIa species of Rpb1p and with Rpb1p that is phosphorylated on some, but not all, repeats. The decrease in mobility of the 8WG16-reactive species observed in Fig. 1A is indicative of an increase in overall phosphorylation of the CTD. Taken together, the results presented in Fig. 1A indicate that deletion of the CTK1 gene leads to an increase in serine 5 phosphorylation. Fig. 1A also indicates a decrease in the mAb H5-reactive epitope in the ctk1Δ strain. In this figure we observe an approximately 2-fold reduction, but the magnitude of the decrease is dependent on growth state (see below). This result suggests that CTDK-I may be involved in phosphorylation of Ser5.

To determine whether all of the RNA polymerase II is released from the cells during grinding with glass beads, we compared the protein in the cell extracts with protein remaining in the pellet containing cell debris. Fig. 1B shows that the H14- and H5-reactive forms of pol II are completely extracted while some of the heat shock protein Ssa1p remains in the pellet.

To control for the possibility that changes in CTD phosphorylation were due to secondary genetic changes in the *ctk1Δ* strain, a T7 promoter sequence such that the PCR reaction can be used as template to synthesize the antisense probe. The primers used for the PCR are as follows: SSA4, 5′-GAATCGACTGAATCGTACCGCGCGG-3′ and 5′-TAATAGCCTACATAGGGCC TCTTCCGCAACG-3′; SSA5, 5′-TAATAGCCTACATAGGGG GCTACCGTG TGGGAACTTT-3′; GSY7, 5′-TCCCGTGACCTAAAGCAGCAG-3′ and 5′-TAATAGCCTACATAGGGG GCTACTGCG TGGGAACTTT-3′; CTT1, 5′-TAAATAGCCTACATAGGGG GCTACCGTG TGGGAACTTT-3′ and 5′-TAATAGCCTACATAGGGG GCTACTGCG TGGGAACTTT-3′.

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cells, we re-transformed these cells with a plasmid expressing wild-type CTK1. In Fig. 1C we see that this plasmid restores wild-type levels of reactivity with both mAb H14 and H5. Thus, the effects seen are specific to CTK1. We also observe a marked decrease in H5 reactivity in ctk1Δ cells in this experiment compared with the experiment shown in Fig. 1A. In Fig. 1C the cells were grown in minimal media lacking leucine to select for the CTK1-containing plasmid. Growth in minimal media enhances the effect of ctk1Δ on the H5-reactive epitope.

Growth-related Changes in CTD Phosphorylation in ctk1Δ Cells—In our previous studies we showed that phosphorylation of different CTD phosphoacceptor sites is independently regulated. Both nutritional limitation and heat shock result in higher levels of Ser² phosphorylation with little change in Ser⁵ phosphorylation (39). To examine growth-related changes in ctk1Δ cells, we made protein extracts from CTK1 (ADH6–1a) and ctk1Δ (ADH6–1b) cells at different stages of growth. A typical growth curve was obtained from cells grown in rich media (Fig. 2A). As described earlier (37) ctk1Δ cultures display slow growth but eventually reach stationary phase.

Fig. 2B shows Western blots of protein extracts derived from the cultures described in Fig. 2A. As we described earlier, there is a marked increase in reactivity with mAb H5 as cells approach stationary phase (39). The timing of this increase coincides with the beginning of the “diauxic shift” that occurs upon depletion of glucose and involves major reprogramming of the pattern of gene expression (48). This transient increase in mAb H5 reactivity is not seen in ctk1Δ cells when they reach the same point in the growth curve (Fig. 2B). However, CTK1 deletion does not entirely eliminate Ser² phosphorylation; some reactivity with mAb H5 remains, and this reactivity declines in a similar fashion as both wild-type and mutant cells enter stationary phase.

As shown earlier in Fig. 1, there is a severalfold increase in reactivity with mAb H14 at time points prior to the beginning of stationary phase. This increase is not due to changes in the concentration of Rpb1p as can be seen from immunoreactivity with antibody raised against a peptide corresponding to the amino-terminal nine amino acids of Rpb1p (Anti-JC20). This antibody detects approximately the same amount of Rpb1p in CTK1 and ctk1Δ cells at similar points in the growth curve. In both strains, the total amount of pol II subunit declines as cells reach stationary phase, an observation consistent with the known reduction in overall transcription as cells approach stationary phase (49). With both anti-JC20 and 8WG16 we observe an increase in the slower mobility form of Rpb1p in ctk1Δ cells. This is most obvious for 8WG16 where the reactive band in ctk1Δ cells is well above the 200-kDa marker, whereas that in the CTK1 cells is even with the marker. Antibody against heat shock protein Ssa1p was used to show that equal amounts...
of protein were extracted from cells at various points in the growth curve.

CTK1-dependent Expression of Diauxic Phase Genes—The absence of inducible H5-reactive CTD epitope during diauxic shift in ctk1Δ cells suggests that this form of pol II may be involved in regulating genes that are induced in this phase of the growth cycle. We have examined expression of glycogen synthase encoded by the GSY2 gene (50) and cytosolic catalase encoded by the CTTL1 gene (51). Both of these genes have been shown to be induced during the diauxic shift (48). In Fig. 3 we show that in wild-type cells, expression of GSY2 and CTTL1 is induced early in the diauxic shift, decreases, and then gradually increases as cells reach stationary phase. In contrast, in ctk1Δ cells expression of neither GSY2 nor CTTL1 is induced but rather gradually increases during growth with maximum expression reached after the diauxic shift. This maximum level is markedly lower than that seen in CTK1 cells during the diauxic shift.

ctk1Δ Effects on CTD Phosphorylation and Heat Shock Gene Expression—As we have previously shown, heat shock leads to an increase in the mAb H5-reactive CTD epitope (39). In Fig. 4A we show that this same increase in mAb H5 reactivity is maintained in the ctk1Δ background. We also observe that induction of heat shock proteins Ssa3p and Ssa4p occurs in both the mutant and wild-type backgrounds. Together, these results indicate that CTDK-I is not required for the heat shock response. Interestingly, some expression of Ssa3p/Ssa4p is observed even in the absence of heat shock (Fig. 4A), and Northern blot analysis (Fig. 4B) confirms that SSA3/SSA4 transcripts are observed during growth at 30 °C only in the ctk1Δ background. Thus, CTDK-I would appear to be involved in regulation of heat shock gene expression under normal growth conditions. The effect of ctk1Δ on SSA4 expression is specific; ACT1 expression is unchanged in the ctk1Δ background, whereas expression of ENO1, which has previously been shown to be sensitive to CTD truncation (52), is reduced.

DISCUSSION

The pattern of CTD phosphorylation is a product of combined action of both CTD kinases and CTD phosphatases. Deleting a CTD kinase gene would be expected to upset the dynamic balance between phosphorylation and dephosphorylation and lead to changes in the CTD phosphorylation pattern. In the present study we have used anti-phospho-CTD monoclonal antibodies to show that deletion of CTK1 changes the pattern but does not eliminate phosphorylation of the yeast pol II CTD. This result is consistent with the existence of multiple CTD kinases. The changes we observe in the ctk1Δ background are in part dependent on growth state and suggest diverse roles for CTDK-I in regulation of CTD phosphorylation.

CTDK-I and CTD Phosphorylation during Logarithmic Growth—The most unexpected result presented here is that the phosphorylation state of the CTD in logarithmically growing cells is increased in the ctk1Δ background. This result suggests that CTDK-I plays a role in negatively regulating CTD phosphorylation during log phase growth. The log phase increase in CTD phosphorylation is specific for Ser2 with little change observed in Ser5 phosphorylation. Both Cdk7 (Kin28) and Cdk8 (Srb 10) kinases have been shown to preferentially phosphorylate Ser2 (20, 53–56). One possibility is that CTDK-I may negatively regulate both of these kinases, perhaps through phosphorylation. An alternative explanation for the increase in Ser2 phosphorylation in ctk1Δ cells is that CTDK-I may positively regulate a CTD phosphatase. Fcp1 is the only known CTD phosphatase (58, 59), but it is not known if its activity is controlled by phosphorylation. In addition, we do not know whether Fcp1 phosphatase is selective for either phosphorylated Ser2, Ser5, or both.

CTK1 deletion could also lead to an increase in Ser5 phosphorylation by a mechanism involving CTD phosphorylation. For example, if CTDK-I were to phosphorylate the CTD in such a fashion that the phosphoacceptors for other CTD kinases were blocked, then we would observe an increase in CTD phosphorylation upon CTK1 deletion. In this scenario CTDK-I would have to phosphorylate the CTD to low density, whereas CTD kinases that function in the absence of CTDK-I would need to phosphorylate the CTD to high density.

CTD Phosphorylation and Growth—We have previously shown that phosphorylation of Ser2 transiently increases late in logarithmic growth (39). The timing of this change in CTD phosphorylation corresponds to the beginning of the diauxic shift that occurs when cells growing in glucose-based medium deplete the glucose and shift from fermentation to respiratory metabolism (60). In the present study we show that this increase in Ser2 phosphorylation does not occur in ctk1Δ cells. The most straightforward explanation is that CTDK-I is responsible for phosphorylating Ser2 during the diauxic shift. We cannot, however, rule out less direct mechanisms in which...
CTDK-I may positively regulate an Ser²-specific kinase or negatively regulate an Ser²-specific phosphatase. Clearly, CTDK-I is not the only kinase capable of phosphorylating Ser² as some reactivity with mAb H5 remains in the ctk1Δ strain, and this reactivity increases during heat shock.

The diauxic shift is accompanied by widespread change in expression of genes involved in carbon metabolism, protein synthesis, and carbohydrate storage (48, 60). Several classes of genes co-regulated during the diauxic shift were identified in a DNA microarray survey (48). One particularly interesting group displays an average 10-fold increase in message levels during early diauxic shift. This class includes the predominantly expressed glycogen synthase gene GSY2 (50) and the cytosolic catalase gene CTTI (51). In this paper we show that the expression pattern for GSY2 and CTTI in wild-type cells correlates with the expression of H5-reactive epitope. Maximum expression is observed during early diauxic shift, and steady-state levels of RNA decline as cells approach stationary phase. In contrast, in ctk1Δ cells GSY2 and CTTI expression is not induced, and only modest increases are observed well after the diauxic shift. This is the first example of a gene that is dependent on CTDK-I for correct regulation. The similar timing of appearance of the H5-reactive epitope and the induction of GS² and CTTI mRNA accumulation further suggests a possible role for Ser² phosphorylation in the increase in steady-state mRNA levels observed during the diauxic shift.

A Gene-specific Role for CTDK-I—The results presented here suggest that CTDK-I is not a general elongation factor but rather functions in a gene-specific fashion. We have identified a class of yeast genes expressed in late log phase as potential targets of regulation. The absence of diauxic phase Ser² phosphorylation and the coincident failure to increase expression of GSY2 and CTTI suggest that expression of these genes is controlled through specific changes in phosphorylation of the CTD by CTDK-I. This is an apparently specific function of CTDK-I as heat shock-induced expression, which is also accompanied by an increase in Ser² phosphorylation, is not affected by CTK1 deletion (Fig. 4).

Our observations together with published observations about the role of CTDK-I in elongation favor a model in which Ser² phosphorylation regulates transcription elongation on specific genes. In this model CTDK-I is attracted to specific transcription complexes in response to regulatory signals. Phosphorylation of the CTD at Ser² by CTDK-I either at the time of promoter clearance or later in elongation establishes an efficient transcription elongation complex leading to accumulation of mRNA.

Involvement of CTDK-I could be triggered by factors bound at the promoter or by cis elements present in the transcribed regions of responsive genes. The promoters of GSY2 and CTTI share several cis-acting promoter elements including the stress response element (61). If factors that bind to the promoter in response to impending glucose depletion can attract CTDK-I, the ensuing CTD phosphorylation could allow for more efficient clearance from the promoter. This may involve breaking contacts between the CTD and components of the preinitiation complex or could be due to the establishment of contacts between the newly phosphorylated CTD and components of the elongation complex. Alternatively, genes regulated by CTDK-I at the diauxic shift may share common cis-acting RNA elements similar to the human immunodeficiency virus transcription- responsive region RNA (TAR) site. TAR binds the activator Tat which in turn recruits P-TEFb which is thought to phosphorylate the CTD leading to productive elongation (30, 62–64). Whether such cis-acting elements are present in yeast diauxic-specific genes is not known.
