Mutual Targeting of Mediator and the TFIIH Kinase Kin28

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In Saccharomyces cerevisiae, Kin28 is a member of the cyclin-dependent kinase family. Kin28 is a subunit of the basal transcription factor holo-TFIIH and its trimeric sub-complex TFIIK. Kin28 is the primary kinase that phosphorylates the RNA polymerase II (RNA pol II) C-terminal domain (CTD) within a transcription initiation complex. Mediator, a global transcriptional co-activator, dramatically enhances the phosphorylation of the CTD of RNA pol II by holo-TFIIH in vitro. Using purified proteins we have determined that the subunits of TFIIK are sufficient for Mediator to enhance Kin28 CTD kinase activity and that Mediator enhances phosphorylation of a glutathione S-transferase-CTD fusion protein, despite the absence of multiple Mediator and/or TFIIH interactions with polymerase. Mediator does not stimulate the activity of several other CTD kinases, suggesting that the specific enhancement of TFIIH kinase activity results in Kin28 being the primary CTD kinase at initiation. In addition, we have found that Kin28 phosphorylates Mediator subunit Med4 in an assay, including purified holo-TFIIH, and either Mediator or recombinant Med4 alone. Furthermore, Kin28 appears to be, at least in part, responsible for the phosphorylation of Med4 in vitro. We have identified Thr-237 as the site of phosphorylation of Med4 by Kin28 in vitro. The mutation of Thr-237 to Ala has no effect on the growth of a yeast strain under normal conditions but confirms that Thr-237 is also the site of Med4 phosphorylation in vivo.

TFIIH is a RNA pol II general transcription factor that, when purified to homogeneity, is composed of nine subunits conserved from yeast to humans. An intact nine-subunit complex, holo-TFIIH, is fully capable of satisfying the TFIIH requirements for transcription in purified systems derived from yeast and mammalian cells (1, 2). In the yeast Saccharomyces cerevisiae the genes encoding all nine subunits are essential for viability. Structurally, TFIIH can be divided into sub-complexes that may have distinct functional roles in the cell. “Core” TFIIH consists of Rad3, Tbh1, Tbh2, Ssl1, and Tbh4, as well as a sixth subunit, Ssl2, that is loosely bound to the core and is often sub-stoichiometric in purified TFIIH. Tbh3, Ccl1, and Kin28 make up a trimeric holo-TFIIH sub-complex called TFIIK (3). In S. cerevisiae, Kin28 and Ccl1 are a cyclin-dependent kinase (cdk)-cyclin-like pair that possesses the ability to phosphorylate the CTD of RNA pol II, whereas in higher eukaryotes the cdk-cyclin (Cdk7/cyclin H) components of TFIIH also serve as a Cdk-activating kinase. It has recently been shown that TFIIK exists separately from holo-TFIIH in yeast extracts, but it is still unclear what function TFIIK, as an independent entity, has in vivo (4). Despite extensive studies (for review see Ref. 5), clearly defining the role of the kinase activity of Kin28 in transcription has remained elusive.

The kinase activity of Kin28 is essential for viability (6), and a whole genome analysis, using the temperature-sensitive mutant strain kin28-ts3, has shown a decrease in the mRNA levels of nearly all RNA pol II-transcribed genes at the non-permissive temperature (7). It is unclear, however, whether this decrease results from defects at transcription initiation or one of the many other processes regulated and targeted by the dynamic phosphorylation of the CTD of RNA pol II. Kin28 has been shown to be the primary CTD kinase at initiation in vivo (6, 8–10), but the CTD and its phosphorylation state also play an important role in transcription elongation, mRNA processing, and mRNA export (11), all of which could affect steady-state mRNA levels. Small molecules that specifically inhibit the kinase activity of Kin28 cause a decrease in elongating polymerases in vivo and a defect in transcription in yeast nuclear extracts (6). However, the role of Kin28 in transcription is not straightforward, because basal transcription reactions reconstituted from purified factors require neither the Kin28 kinase activity nor the CTD itself (12, 13).

Srb10/11 is a second cdk-cyclin-like pair in yeast that is a component of transcription initiation complexes formed in nuclear extracts (14). In an otherwise wild-type background, Srb10/11, which are encoded by non-essential genes, do not seem to be major contributors to the phosphorylation (6, 15) of the CTD in vivo. Genetic and genomic analyses show that Srb10/11 can have both negative (7) and positive (16) effects on the expression of certain subsets of genes in vivo. Experiments in vitro have shown that repression of transcription may originate from Srb10/11 phosphorylating the CTD prior to initiation and preventing the entry of RNA pol II into initiation complex, whereas Kin28 seems to phosphorylate the CTD only after the initiation complex has formed (17). Transcription in nuclear extracts was actually decreased by the specific inhibition of Srb10, but only in the absence of Kin28 kinase activity (6). What causes Kin28 and not Srb10/11 to be the primary CTD kinase at initiation is still an open question. Biochemical
genetic and studies have provided evidence that the role of CTD phosphorylation in transcription is linked to the global co-activator complex Mediator.

The Mediator complex is a conserved interface between gene-specific regulatory proteins and the general transcription apparatus of eukaryotes at transcription initiation (18) and is required for the expression of nearly all RNA pol II-transcribed genes (7). A genetic connection between Mediator and the CTD was revealed by studies that identified extragenic suppressors (the Srb genes) of a cold-sensitive phenotype caused by RNA pol II CTD truncation (19, 20). When Mediator, and Mediator-containing complexes, were subsequently isolated biochemically, Srb2, Srb4, Srb5, Srb6, and Srb7 were all identified as part of the 20-subunit minimal Mediator that is sufficient for activated transcription in a purified system (21, 22). The recessive suppressors Srb8, Srb9, Srb10, and Srb11 form a subcomplex (23) that seems to associate with the 20-subunit complex in certain holopolymers (a pre-formed complex minimally containing Mediator and RNA pol II) preparations and in pre-initiation complexes (14). Biochemical studies have also revealed an intimate physical and functional association between Mediator and the CTD. Purified S. cerevisiae Mediator binds directly to a GST-CTD fusion protein (22, 24), and the CTD, although not required for basal transcription, is required for Mediator to function as a co-activator in the purified transcription system (22). Genetic and functional biochemical studies have also identified connections between TFIHH and Mediator. Deletion of SIN4, whose product protein is a subunit of Mediator, is synthetically lethal with a kin28 temperature-sensitive mutant (25). Assembly of TFIHH into a pre-initiation complex in extracts requires Mediator (26) and, subsequent to transcription initiation, phosphorylation of the CTD by Kin28 correlates with the release of Mediator from the CTD of RNA pol II as promoter clearance occurs (27). Within a purified pre-initiation complex, Kin28 was found to phosphorylate the Med4 and Rgr1 subunits of Mediator (6). In addition, Srb10 has also been shown to phosphorylate Med2 (28). Intriguingly, Mediator has been shown to substantially enhance the phosphorylation of the CTD of RNA pol II by TFIHH, adding a direct functional connection between TFIHH, the CTD, and Mediator.

Several participants in transcription initiation in both yeast and metazoan cells have been shown to enhance phosphorylation of the CTD of RNA pol II. Assembly of the general transcription factors at a promoter (29), DNA alone (30), and TFIIE (31, 32) have all been shown to enhance phosphorylation of the CTD by TFIHH. Mediator purified from mouse (33), Schizosaccharomyces pombe (34), or S. cerevisiae (22) enhances CTD phosphorylation by TFIHH in a largely species-specific manner (34). The potential role of various Mediator components in this phenomenon is unclear, because the Mediator subunit Gal11 alone can enhance TFIHH kinase activity (22), yet purified mutant yeast Mediator lacking Gal11 still enhances CTD phosphorylation at wild-type levels (35). To gain further insight into the Mediator enhancement of CTD phosphorylation, we have defined the substrate and kinase requirements by using minimal substrates and TFIHH sub-complexes that contain Kin28 or other kinases that are capable of CTD phosphorylation. In the course of this work we found that Kin28 phosphorylation of Med4 occurs in vivo as well as in reactions using purified proteins. We have identified Thr-237 as the site on Med4 phosphorylated by TFIHH and investigated the functional role of this modification.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—The S. cerevisiae strain YOT63 (3) with TAP-tagged TFB3 was grown at 30 °C to an A600 of 3–4 in YPD media (2% w/v peptone, 1% w/v yeast extract, and 2% glucose). The following steps were then performed at 4 °C. The cells were harvested by centrifugation, resuspended, broken with glass beads as described previously (22), and the lysate was clarified by centrifugation in a Beckman JLA-10.500 at 8000 rpm for 20 min. One-ninth volume of 5 m potassium acetate was added, and the lysate was stirred at 4 °C for 10 min. Polyethyleneimine was slowly added to a final concentration of 0.2%, and the lysate was stirred for 30 min followed by a 2-h, 35,000 rpm ultracentrifugation step in a Beckman 45Ti supernatant. The protein concentration of the supernatant was measured with the Bio-Rad Protein Assay, and 600 mg of protein was loaded on to 100 μl of Ig-Sepharose resin (Amersham Biosciences) equilibrated in IPP-550 (IPP buffer contained 20 mM Tris acetate, pH 7.8, 0.1% Nonidet P-40, 1 mM DTT); the millimolar concentration of potassium acetate is indicated by a hyphen followed by a number) with protease inhibitors (36). The suspension was incubated for 1 h with rotation. The beads were collected in a column and washed with 10 ml of IPP-550 with protease inhibitors, 10 ml of IPP-550, and 10 ml of IPP-550 with 0.5 mM EDTA. The beads were resuspended in 1 ml of IPP-550 with 0.5 mM EDTA, and 20 μg of mutant TEV protease that resists autoproteolytic inactivation (purified according to Lucast et al. (37)) was added. The beads were incubated with the TEV protease buffer for 10–15 h and the TEV eluate drained from the column. 3 μl of 1% calcium chloride was added to the eluate, and the mixture was incubated on 200 μl of calmodulin-affinity beads (Stratagene) equilibrated in CBB-550 (CBB buffer contained 20 mM Tris acetate, pH 7.8, 0.1% Nonidet P-40, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 μg chloroform with chloroform for 1 h with rotation. The resin was washed three times with 10 ml of CB-550. Bound proteins were eluted with 5 × 200 μl CBB (20 mM Tris acetate, pH 7.8, 200 mM potassium chloride, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 0.1% Nonidet P-40, and 2 mM EDTA). To remove any remaining holo-TEFIIH from the TFIHK, the sample was adjusted to 100 mM potassium acetate and loaded on a Mono Q 5/5 column (Amersham Biosciences) equilibrated in Buffer A-100 (Buffer A contained 20 mM Tris acetate, pH 7.8, 10% glycerol, 0.01% Nonidet P-40, 1 mM DTT; protease inhibitors; the millimolar concentration of potassium acetate is indicated by a hyphen followed by a number) and eluted with a 18-ml linear gradient to A-1000. TFIIK eluted at ~260 mM potassium acetate.

The vector for expression of recombinant Med4 with a 6-histidine tag (His6 Med4) on its N terminus was constructed by amplifying the S. cerevisiae Med4 gene by PCR using the primers 5′-GGAATTCATATGTCAGCAGAAGACTAAGGAAA-3′ and 5′-GGCGCCGAGATCTCTTTCGATTTGGCTGG-3′, cloning the PCR product with Ndel and Xhol and ligating the vector into the expression vector pET15b (Novagen). The vector (pMed4-15b) was transformed into BL21(DE3) cells and expressed at 37 °C. The cells were harvested and lysed with a French pressure cell, and the lysate was purified from the insoluble fraction on a 50-mL-nitritolactic acid (Qiagen) following the manufacturer’s protocols.

To construct a GST-CTD expression vector, the C-terminal domain and the preceding acidic domain (38) of S. cerevisiae Rpb1 was amplified by PCR using the primers 5′-GGCGCGGATCCACGTGTTGGCGACCTGTCAGGTCCAGTTTCTCTGTCTGGG-3′ and 5′-GGCGCCGAGATCTCTTTCGATTTGGCTGG-3′, cloning the PCR product with Ndel and Xhol and ligating the vector into the expression vector pET15b (Novagen). The vector (pMed4-15b) was transformed into BL21(DE3) cells and expressed at 37 °C. The cells were harvested and lysed with a French pressure cell, and the lysate was purified from the insoluble fraction on a 50-mL-nitritolactic acid (Qiagen) following the manufacturer’s protocols.

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tic peptides to locate the exact position of either phosphoserine with the computer-generated fragment ion series of the predicted tryp-

Western blot analysis—To examine the phosphorylation state of Med4 in the wild-type strain (GP260-2) and Kin28Δctd temperature-sensitive strain (JGV105) we followed the protocol of Valay et al. (8) previously used to harvest and lyse these same strains for the analysis of the phosphorylation state of Rpb1p. In brief, yeast cultures were grown in YPD medium at 24 °C to an A600 of ~1.0, quickly shifted to 37 °C, and 5-ml samples were removed at 0, 20, and 60 min. For analysis of the med4-T237A mutant and the MED4 wild-type control, the cells were grown in YPD medium to an A600 of ~1.2 at 30 °C. The cells were harvested and disrupted as described (8) and resuspended in 200 μl of loading dye. Protein samples of ~2 μl were loaded onto an 8% (w/v) polyacrylamide gel, transferred to polyvinylidene difluoride membrane (Amersham Biosciences), and probed with polyclonal α-Med4 serum.

Genetic manipulations—A BamHI-Xhol fragment containing the MED4 gene and its native promoter was cut out of pGM23 (22) and cloned into these same sites in pRS315 (40) to construct the vector pMED4 (LEU2 MED4). Starting from pMED4 the Stragante QuickChange kit was used to mutate Thr-237 to alanine to create pMED4 T237A (LEU2 med4-T237A), or a stop codon to create pMED4 Δ237 (LEU2 med4-Δ237).

A diploid yeast strain (accession number Y22430, EUROSCARF, Frankfurt), that was heterozygous for MED4 deletion (BY4743; MAT a/a; his3Δ1/his3Δ1; leu2Δ2α/leu2Δ2α; lys2Δ2α/lys2Δ2α; MET15/.met15Δ30; ura3Δ0/ura3Δ0; med4.4::kanMX4/MED4) was transformed with pGM23 (MED4 URA3) and sporulated, and tetrads were dissected on YPD agar and scored for survival on 5-fluoroorotic acid (5-FOA) and G418. Using a spore that was G418-resistant and subject to killing by 5-FOA (MATa; his3Δ1; leu2Δ2α; lys2Δ2α; met15Δ30; ura3Δ0; med4.4::kanMX4; pGM23 (MED4 URA3)), the pMED4 (LEU2 MED4), pMED4 T237A (LEU2 med4-T237A), or pMED4 Δ237 (LEU2 med4-Δ237) plasmids were shuffled in the previous study (41) and survival was scored on 5-FOA.

Mass spectrometry—Gel-resolved proteins were digested with trypsin, batch-purified on a reversed-phase (RP) micro-tip, and an aliquot analyzed by matrix-assisted laser desorption/ionization (MALDI) reflectron time-of-flight (TOF) mass spectrometry (MS) (UltraFlex TOF/TOF; BRUKER Daltonics; Bremen, Germany) for peptide mass fingerprinting, as described (42, 43). This served to confirm the identity of the peptides and to locate possible differences between the trypptic peptide maps of the phosphorylated and unphosphorylated forms. The remainder of the RP-eluted digest mixtures were then subjected to immobilized gallium(III) affinity chromatography for selective capture of phosphopeptides, followed by elution with phosphate buffer, desalting over an RP column, and a second round of MALDI-reflectron TOF MS (44). Peak m/z values were matched to the protein sequence, allowing for the likely presence of one or more phosphate groups. Mass spectrometric sequencing of the putative phosphopeptides was then carried by MALDI-TOF/TOF (MS/MS) analysis using the UltraFlex instrument in “LIFT” mode. Fragment ion spectra were inspected for a', b', and y' ions to compare with the computer-generated fragment ion series of the predicted tryptic peptides to locate the exact position of either phosphoserine or phosphothreonine.

RESULTS

A GST-CTD Fusion Protein Is Sufficient as a Substrate for Mediator-enhanced TFIIF Kinase Activity—Both the CTD (21, 22) itself and the core structure of RNA pol II (45) have been implicated as being important for the interaction of Mediator with the polymerase. Additionally, it has been found that TFIIFII physically interacts directly with RNA pol II (46). This finding suggests that the polymerase is a slightly better substrate for the TFIIF kinase than the CTD alone. The magnitude of Mediator enhancement of TFIIFII kinase activity, however, was not markedly changed by small increases in the amount of RNA pol II substrate.

TFIIK Contains the Molecular Information Required for Enhancement of CTD Kinase Activity by Mediator—To determine if the TFIIK sub-module of holo-TFIIH retained all of the components required for Mediator-enhanced Kin28 kinase activity, we isolated TFIIK utilizing a modified version of the TAP purification described by Kornberg and colleagues (3). To assure that there were no sub-stoichiometric containing amounts of holo-TFIIH, we applied the TAP-purified TFIIK to a Mono Q column. TFIIK elutes from Mono Q at a much lower salt concentration (260 mM potassium acetate) than holo-TFIIH (750 mM potassium acetate) (48). A silver stain gel shows the final homogeneous TFIIK (Fig. 2A). A Western blot (Fig. 2B) shows the absence (to within the level of detectability of the Western blot) of the Tbh1 subunit in the TFIIK preparation as compared with the holo-TFIIH preparation used in Fig. 1. The Western blot was also used to normalize the amount of the kinase subunit (Kin28) when comparing holo-TFIIH and TFIIK for Mediator-enhanced kinase assays. Using the GST-CTD as a substrate we compared the kinase activity of holo-TFIIH and TFIIK in the presence or absence of Mediator. Mediator increased the kinase activity of TFIIK with a similar -fold enhancement to that of an equimolar amount of holo-TFIIH (Fig. 3). This finding indicates that the core subunits of TFIIH are unnecessary for Mediator enhancement of Kin28 kinase activity. Holo-TFIIH appears, in this assay, to have a slightly higher specific activity than TFIIK. It is unclear, however, whether this is an intrinsic property of the complex or...
Kin28 and Mediator

Mediator Exhibits Specificity for Enhancement CTD Phosphorylation by TFIIH—In addition to the TFIIH kinase Kin28, there are many other kinases that can phosphorylate the CTD. Among the additional kinases in yeast that can phosphorylate the CTD in vitro are CTK1, Bur1, and Srb10/11 (5). CTK1 and Bur1 appear to be involved in phosphorylation of the CTD during elongation and promote elongation (49) and polyadenylation (50), respectively. Neither CTK1 nor Bur1 is part of a pre-initiation complex assembled in nuclear extracts (6). Casein kinase 1 will also phosphorylate the CTD in vitro with sufficient specificity for Ser-5 to recruit the capping enzyme (51). Kin28 appears to be the primary CTD kinase at initiation of transcription (9, 10). To investigate whether Mediator contributed to this effect by specifically stimulating TFIIK kinase activity, we tested both purified casein kinase 1 and rSrb10/11 for Mediator-enhanced CTD kinase activity. The amounts of the two kinases used were normalized to give an equal amount of kinase activity most likely involves specific interactions with TFIIK rather than presenting the substrate in a favorable orientation for phosphorylation.

Assembly into the Mediator Complex Is Not a Prerequisite for TFIIH/TFIIK Phosphorylation of Med4—When performing the Mediator-enhanced kinase assays we observed a band of ~38 kDa that was phosphorylated when Mediator and TFIIH/TFIIK were present, regardless of the presence or absence of the CTD substrate (Fig. 5B). Neither rSrb10/11 nor casein kinase 1 phosphorylated a protein corresponding to this molecular weight (data not shown). The above data indicated that TFIIK is directly phosphorylating a subunit of Mediator. The molecular weight of this band and an earlier report (39) that Med4 was a phosphoprotein led us to suspect that Med4 was the subunit being phosphorylated. A report by Hahn and colleagues, while this work was in progress, supported this hypothesis by demonstrating that Kin28 could phosphorylate Med4 in a purified pre-initiation complex (6). This same report demonstrated that Kin28 also phosphorylated the Rgr1 subunit of Mediator. Interestingly we have not observed TFIIH/TFIIK phosphorylation of a band of the molecular weight of Rgr1 in our system composed only of purified TFIIH and Mediator (data not shown), suggesting perhaps that an alternative Mediator conformation (45) or additional factor may be required for Rgr1 phosphorylation by Kin28. To determine whether TFIIK could phosphorylate Med4 only in the context of Mediator, whether the other members of the complex were dispensable for phosphorylation of Med4 by TFIIH, we expressed and purified recombinant yeast Med4 protein from Escherichia coli. By placing a 6-histidine tag on the N terminus of Med4, when isolated from yeast, is present in a 1:1 molar ratio of the complete Med4 open reading frame (His$_6$-rMed4) we were able to express and isolate milligram quantities of soluble Med4 using a one-step purification on nickel-agarose (Fig. 5A). Med4, when isolated from yeast, is present in a 1:1 molar ratio with the rest of the Mediator complex (22). Using an equimolar amount of His$_6$-rMed4 and Mediator as substrates, we examined TFIIK phosphorylation of Med4. Regardless of whether it is present as a subunit in Mediator or not, TFIIK phosphorylates Med4 to an equal degree (Fig. 5B). Adding increasing amounts of the His$_6$-rMed4 in the kinase reactions shows that TFIIK is not limiting in these reactions.

Kin28 Phosphorylates Med4 in Vivo—Even though a recent study demonstrated that Kin28 can phosphorylate Med4 in purified pre-initiation complexes, it was still unclear whether Kin28 phosphorylates Med4 in vivo. Med4 has been shown to exist mostly in a phosphopeptide form when Mediator is isolated under conditions that prevent dephosphorylation (39).
Essential Function in Vivo—S. cerevisiae MED4 gene is essential for viability (22). To determine whether phosphorylation on Thr-237 was required for its essential function we mutated Thr-237 to alanine (T237A). Alignment of MED4 orthologues from diverse eukaryotic species (34) showed that Thr-237 falls in a largely non-conserved region from residue 210 to the C terminus at residue 284. To ascertain if the C

32P-labeled ATP) described in Fig. 5B for the 3000-fmol reaction and increased the reaction time to 10 h to ensure as complete phosphorylation as possible. An analytical scale reaction (using 32P-labeled ATP) run in parallel estimated that ~75% of the substrate was phosphorylated (assuming phosphorylation at a single site). The bands corresponding to phosphorylated and non-phosphorylated rMed4 were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. Peptide patterns served to confirm identity of the proteins and were then compared for differences. Two m/z peaks, at 1793.79 and 1921.90 atomic mass units, were observed in the spectra of “rMed4-PO4” that were absent from the “rMed4.” The m/z values mapped to predicted, largely overlapping, monophosphorylated fragments of the Med4 sequence (EGTPKTDSSFIFDGTAK and KEGTPKTDSSFIFDGTAK) with mass discrepancies of less than 20 ppm. Next, the same two peptides were selectively retrieved by immobilized gallium(III) affinity chromatography (44) and reanalyzed by MALDI-TOF/TOF MS/MS sequencing. The presence of several unique fragment ions confirmed the peptide identities and allowed us to map the single phosphate group to Thr-237 (Fig. 7A).

Phosphorylation of Med4 on Thr-237 Is Not Required for Its Essential Function in Vivo—The S. cerevisiae MED4 gene is essential for viability (22). To determine whether phosphorylation on Thr-237 was required for its essential function we mutated Thr-237 to alanine (T237A). Alignment of Med4 orthologues from diverse eukaryotic species (34) showed that Thr-237 falls in a largely non-conserved region from residue 210 to the C terminus at residue 284. To ascertain if the C

This same work also established that the two bands observed in α-Med4 Western blots represented the phosphorylated (low mobility) and non-phosphorylated (high mobility) forms of the protein. To investigate Kin28 phosphorylation of Med4 in vivo, we adapted the methodology used to monitor CTD phosphorylation by Kin28 in vivo (8). Using the mobility shift of Med4 (39) as a diagnostic, we monitored the phosphorylation of Med4 in wild-type cells and in mutant kin28-ts3 cells grown at 24 °C or shifted to the non-permissive temperature (37 °C) for up to 60 min. At the point of the temperature shift we observe that virtually all of Med4 is present in the lower mobility phospho-protein form in both strains (Fig. 6A). Upon shifting to the non-permissive temperature, the higher mobility form of the protein begins to accumulate in the mutant and its amount increases with time (Fig. 6A). In contrast, the wild-type cells maintain a constant level of the Med4 phosphoprotein and the higher mobility form cannot be detected. We conclude that Kin28 is, at least in part, responsible for maintaining Med4 phosphorylation in vivo. It is unclear whether the remaining phospho-Med4 in the kin28-ts3 cells after an hour at the non-permissive temperature reflects slow dephosphorylation and/or turnover of Med4, or whether there are additional kinases contributing to its phosphorylation. It was observed earlier that purified Mediator often contains two bands recognized by α-Med4 antibodies, corresponding to the phosphorylated and the non-phosphorylated forms of the protein. To confirm the assignment of the two forms of the protein detected in extracts (Fig. 6A), we compared samples of purified Mediator and purified Mediator treated with phosphatase side by side with a mutant extract sample. The data indicate that the low mobility form of Med4 and the high mobility form, which results from phosphatase treatment, correspond to the two forms of the protein identified in extracts (Fig. 6B).

Kin28 Phosphorylates Med4 on Thr-237—To identify the TFIIK phosphorylation site(s) on Med4 we performed a preparative scale phosphorylation of His₆-rMed4 to use for phosphopeptide mapping. To prepare the phospho-Med4, we used the purified components and the conditions (leaving out the
terminus of Med4, regardless of its phosphorylation state, was required for the essential function of Med4 we also mutated Thr-237 to a stop codon. In haploid strains in which the only source of Med4 was supplied by the mutant gene, both the T237A and Thr-237 to stop codon mutant strains were identical to wild-type for growth on rich media at 30 °C (data not shown). To determine if Thr-237 was the site of Med4 phosphorylation in vivo, we grew the T237A strain and a wild-type control in vivo, and studied the protein migration by SDS-PAGE. Fig. 7B shows that the Med4 T237A mutant protein migrates solely as the non-phosphorylated form (Med4) we also loaded a lane of purified Mediator that contained both forms (see Fig. 6B).

It is interesting that Kin28 is specific for phosphorylation of Med4 and that Mediator, in turn, specifically enhances the kinase activity of Kin28. Additional studies will be required to determine if these events are coordinated.

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