Cyclin K Functions as a CDK9 Regulatory Subunit and Participates in RNA Polymerase II Transcription

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Important progress in the understanding of elongation control by RNA polymerase II (RNAPII) has come from the recent identification of the positive transcription elongation factor b (P-TEFb) and the demonstration that this factor is a protein kinase that phosphorylates the carboxyl-terminal domain (CTD) of the RNAPII largest subunit. The P-TEFb complex isolated from mammalian cells contains a catalytic subunit (CDK9), a cyclin subunit (cyclin T1 or cyclin T2), and additional, yet unidentified, polypeptides of unknown function. To identify additional factors involved in P-TEFb function we performed a yeast two-hybrid screen using CDK9 as bait and found that cyclin K interacts with CDK9 in vivo. Biochemical analyses indicate that cyclin K functions as a regulatory subunit of CDK9. The CDK9-cyclin K complex phosphorylated the CTD of RNAPII and functionally substituted for P-TEFb comprised of CDK9 and cyclin T in in vitro transcription reactions.

Accumulating evidence indicates that the expression of many protein coding genes is regulated at the level of transcription elongation. Understanding of elongation control by RNAPII has been hampered by slow progress in the identification of factors involved in transcription elongation. An emerging model is that the interplay of positive and negative elongation factors controls the elongation potential of RNAPII in different promoters (1). Support for this view comes from the recent identification of the negative elongation factors 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity-inducing factor, negative elongation factor, and factor 2 and the positive elongation factor P-TEFb. Factor 2 is an ATP-dependent termination factor that releases transcripts associated with stalled RNAPII molecules (2). DSIF is a repressor of elongation that was identified as a factor that renders in vitro transcription reactions sensitive to the drug DRB (3). NELF works in conjunction with DSIF to repress RNA polymerase II elongation (4). P-TEFb is a DRB-sensitive kinase that is believed to stimulate the elongation potential of RNAPII by phosphorylating the CTD of RNAPII molecules that are engaged in early transcription elongation (5, 6). It was recently suggested that P-TEFb-mediated phosphorylation of the CTD prevents the association of DSIF with RNAPII and thereby overcomes DSIF-dependent repression (7). Although it has been long accepted that CTD phosphorylation plays a critical role in transcription, it has been difficult to ascertain the mammalian kinases responsible for CTD phosphorylation in vivo. The observation that the ability of several drugs to block CTD phosphorylation in vivo correlates with the ability of these compounds to inhibit P-TEFb in vitro strongly suggests that P-TEFb might indeed function as a CTD kinase in vivo (8). Additional evidence showing that P-TEFb kinase functions as a positive elongation factor in vivo comes from studies with the HIV Tat protein that have shown that the catalytic activity of P-TEFb is required for Tat-dependent stimulation of transcription elongation (9, 10).

Native human P-TEFb appears to exists as a polypeptide complex composed of a catalytic subunit (CDK9), a regulatory subunit (cyclin T), and potentially several additional polypeptides that are found in immunoprecipitates isolated with CDK9 antibodies (11–13). The observation that CDK9 can be found in different chromatographic fractions (14) and that two different cyclin genes (cyclin T1 and cyclin T2) can function as CDK9 regulatory subunits (12) suggests that CDK9 might associate with functionally different complexes and thereby participate in different cellular processes. To further characterize the components of CDK9-containing complexes we used the yeast two-hybrid interaction system to identify proteins that associate with CDK9. In this report we show that cyclin K associates with CDK9 in vivo and in vitro and demonstrate that cyclin K is a CDK9 regulatory subunit.

MATERIALS AND METHODS

Yeast Two-hybrid Screening—The yeast two-hybrid screen utilized the Mammalian MATCHMAKER Two-Hybrid Assay Kit (CLONTECH, Palo Alto, CA). The screening and selection of clones was performed as suggested by the manufacturer. The bait was constructed by inserting the full-length CDK9 gene (EcoRI/Sall fragment) into the pAS2 plasmid. A cDNA library made from Jurkat cell RNA cloned in the pACT2 target plasmid (CLONTECH) was used in the screen. The Jurkat library was screened two times in order to confirm screening results. More than 1 × 107 colonies were analyzed in each screen. The cyclin K gene isolated by the yeast two-hybrid screen was missing 20 amino acids at the carboxyl terminus when compared with a previously published sequence. A full-length cDNA clone was generated using a polymerase chain reaction method.

Protein Purification—CDK9-cyclin T1, CDK9-cyclin K, and cyclin K were purified by successive chromatographic steps on nickel-agarose and gel filtration using identical conditions. The nickel-agarose step was performed as described previously (12). Five ml of the material eluting from nickel-agarose (2.5–5.0 mg) was loaded into a Superdex 200 column (Superdex 200 HiLoad 16/60, Pharmacia) equilibrated with 25 mM Hepes, pH 7.9, 500 mM NaCl, 1% Triton X-100, 0.1% Nonidet P-40, 0.1 mM EDTA, and 10 mM β-mercaptoethanol (BME). One 30-ml fraction (corresponding to the void volume) and 90 1.5-ml fractions (included volume) were collected.

CDK9 was obtained from insect cells that were infected with a virus

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1 The abbreviations used are: RNAPII, RNA polymerase II; P-TEFb, positive transcription elongation factor b; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; CTD, carboxyl-terminal domain; HIV, human immunodeficiency virus; BME, β-mercaptoethanol.
target or empty bait plasmids were plated in the experiments in which yeast cells were transfected with either empty were plated and grown in the upper third region of each plate. Control either cyclin T or cyclin K containing target plasmids as indicated in these discrete plate regions. Cells transfected with the CDK9 bait and experiments, a test and two controls, are plated and grown in each of plate is divided into three regions. Yeast cells from three independent

...equilibrated with 25 mM Hepes, pH 7.5, 10% glycerol, 50 unstable in 0.5 M KCl, and most of dcT was removed in the nickel-agarose step (data not shown). The CDK9 nickel-agarose fraction was further fractionated on a Mono Q (HR 5/5, Amersham Pharmacia Bio-...nzyme mixture (Wallac). Plates were read in a microplate

...CTD Kinase Assay—Kinase reactions (50 μl) contained 25 mM Hepes, pH 7.6, 5 mM MgCl2, 5 mM dithiothreitol, 10 μM ATP, 0.3 μCi of [32P]ATP (7,000 Ci/mmol), 10 mM BME, 100 μg/ml bovine serum albumin, 0.1% Nonidet P-40, and a 0.3 mM amount of a biotinylated peptide containing four copies of the consensus CTD heptapeptide YSPTSPS. Reactions were performed on 96-well microtiter plates coated with NeutraAvidin (Reacti-Bind NeutraAvidin plates, Pierce). Reactions were performed on 96-well microtiter plates coated with NeutraAvidin (Reacti-Bind NeutraAvidin plates, Pierce). Reactions were incubated at room temperature for 1 h. At the end of the incubation, reaction mixtures were removed, the plates were washed three times with distilled water and allowed to air dry before the addition scintillation mixture (Wallac). Plates were read in a microplate scintillation counter (Packard).

RESULTS

To further characterize CDK9-containing complexes we decided to employ the two-hybrid protein interaction method to identify CDK9-interacting proteins. We used the full-length CDK9 gene as bait to screen an expression library generated from Jurkat cell cDNA. Three different strong positive genes that were also positive in secondary screens were isolated (see “Materials and Methods”). Sequence analysis of these genes revealed that one of them encoded a novel protein and the other two encoded cyclin T1 and cyclin K, respectively. The isolation of cyclin T1 validated the screen in that it showed the Gal4-CDK9 bait protein was competent for binding to a physiologically relevant CDK9-interacting protein. Cyclin K was recently identified in a genetic screen in yeast by virtue of its ability to complement the lethality associated with the deletion of G1 cyclin genes (14). Interestingly, it was previously reported that cyclin K interacts with RNA polymerase II and that immunoprecipitates obtained with cyclin K antibodies contained CT D and Cdk kinase kinase activity (14). Those studies, however, did not identify the kinase that associates with cyclin K and could not rule out the possibility that contaminating kinases present in the immunoprecipitates were responsible for the observed CT D and Cdk kinase kinase activities.

The interaction between CDK9 and cyclin K in the yeast two-hybrid assay was specific as suggested by the observation that neither the CDK9 bait plasmid, cyclin T1, or cyclin K target plasmids conferred significant growth to yeast cells when transfected independently (Fig. 1). Cyclin T1 and cyclin K scored similarly in the growth selection assay when cotransfected together with the CDK9 bait plasmid (Fig. 1), suggesting that all two proteins interact with CDK9 with similar efficiency in this assay system.

Cyclin K Is a CDK9 Regulatory Subunit

FIG. 1. Cyclin K interacts with CDK9 in a yeast two-hybrid assay. A, schematic representation of yeast two-hybrid assays. Each plate is divided into three regions. Yeast cells from three independent experiments, a test and two controls, are plated and grown in each of these discrete plate regions. Cells transfected with the CDK9 bait and either cyclin T or cyclin K containing target plasmids as indicated in B were plated and grown in the upper third region of each plate. Control experiments in which yeast cells were transfected with either empty target or empty bait plasmids where plated and grown in the bottom right and left, respectively. Growth selection was as described under “Materials and Methods.” B, photograph of Petri dishes from the experiments outlined above after cells were allowed to grow 3–6 days at 30 °C.

FIG. 2. Cyclin K interacts with CDK9 and forms a functional kinase complex. A, fractionation of CDK9-cyclin K complex in a Superdex 200 gel filtration column. The CDK9-cyclin K complex and CDK9 were first purified by chromatography on nickel-agarose as described under “Materials and Methods.” Similar amounts of nickel-agarose-purified CDK9-cyclin K and CDK9 were then fractionated on a Superdex 200 HP gel filtration column. The corresponding elution profiles are shown in panels I and II. Column fractions are indicated at the top of panel I. B, the CDK9-cyclin K complex has CTD kinase activity. Similar amounts of nickel-agarose-purified CDK9-cyclin T1 and CDK9-cyclin K complexes were fractionated in a Superdex 200 column. Column fractions were assayed for CTD kinase activity. Conditions were as described under “Materials and Methods.”
expressing cyclin K or co-infected with both CDK9 and cyclin K expressing baculoviruses. Cyclin K contained a histidine tag at the carboxyl terminus and CDK9 was untagged. Purification of cyclin K on nickel-agarose columns indicated that CDK9 could be readily isolated together with cyclin K from extracts derived from co-infected cells but not from extracts that were infected with the cyclin K expressing virus alone, indicating that this was not endogenous CDK9 (data not shown). The stability of the nickel-agarose CDK9-cyclin K complex was further analyzed by fractionation on a gel filtration column equilibrated in high salt conditions (Fig. 2A). Furthermore, the elution profile of CDK9 in the CDK9-cyclin K complex was almost half of that of CDK9-cyclin T complex but between 10–15 times greater than that of CDK9-cyclin K complex detected by Coomassie staining, we compared its elution profile to that of the human CDK9-cyclin T1 complex and determined that the elution profiles of both complexes could be readily distinguished. Furthermore, the levels of kinase activity recovered from both fractionations were in the same range (2-fold difference), which also argues against the possibility that cyclin T from insect cells could be responsible for the activity associated with the CDK9-cyclin K complex (Fig. 2B).

It was important to quantitate the activity of the CDK9-cyclin K complex and to compare its specific activity to that of the better characterized CDK9-cyclin T1 complex. CDK9 and both kinase complexes were purified from insect cells to near homogeneity as described under “Materials and Methods.” The only difference between each kinase complex is that the CDK9-cyclin T1 complex contains a histidine tag in the CDK9 subunit while the CDK9-cyclin K complex contains a histidine tag at the NH2 terminus of the cyclin K subunit. Expression of CDK9 in the absence of a cyclin subunit yielded very low levels of purified protein, and therefore we decided to co-express CDK9 with *Drosophila* cyclin T, because this cyclin readily dissociates from CDK9 under high ionic strength. Each protein preparation was carefully quantitated (Fig. 3A) and used in dose-response experiments. We found that the specific activity of the CDK9-cyclin K complex was almost half of that of CDK9-cyclin T1 complex but between 10–15 times greater than that of CDK9 alone (Fig. 3B). We cannot rule out the possibility that the small levels of kinase activity detected with CDK9 alone...
might come from contamination with Drosophila cyclin T1. These experiments clearly indicate that binding of cyclin K to CDK9 results in the reconstitution of an active CDT kinase.

The CDK9-Cyclin K Complex Functions in RNA Polymerase II Transcription—We have previously shown that extracts depleted of P-TEFb activity with CDK9 antibodies do not support efficient transcript elongation and that this transcription deficiency can be complemented with the recombinant CDK9-cyclin T1 complex (12). To test whether the CDK9-cyclin K complex was also transcriptionally competent, CDK9-depleted extracts were supplemented with different amounts of each kinase complex and found that CDK9-cyclin K promoted the formation of DRB sensitive transcripts (Fig. 4). The transcription activity of the CDK9-cyclin K complex was lower than that of the CDK9-cyclin T1 complex, and the difference in transcription activity between both kinase complexes seems to be proportional to their kinase activity (Fig. 4). Importantly, CDK-cyclin K-dependent transcription was inhibited by DRB (Fig. 4) and by other drugs previously shown to inhibit P-TEFb kinase activity (data not shown).

DISCUSSION

In this study we provide conclusive biochemical evidence indicating that cyclin K can function as a CDK9 regulatory subunit. This is an unexpected finding because the cyclin box of cyclin K is only 29% identical to the cyclin T1 cyclin box at the amino acid level. Despite the seemingly low sequence similarity, cyclin K is most closely related to human cyclins T1 and T2 among all other known human cyclin genes in the cyclin box region (data not shown). Interestingly, our unpublished observations indicate that the binding of Drosophila cyclin T to CDK9 is weaker than that of cyclin K despite the fact that the sequence similarity of Drosophila cyclin T to human cyclin T1 is much higher (60% identity).

The functional consequence of having multiple regulatory subunits for CDK9 is not yet understood. Thus far we have not found obvious differences in substrate specificity among CDK9 subunits for CDK9 is not yet understood. Thus far we have not found obvious differences in substrate specificity among CDK9 subunits for CDK9-cyclin K has the same sensitivity to a panel of structurally diverse inhibitors of CDK9-cyclin T1 (data not shown). It is also possible that functional differences among recombinant CDK9 complexes cannot be detected in these in vitro assays due to the absence of additional factors involved in kinase function. Such factors might be scaffold proteins that bind to different cyclin subunits and confer selectivity by mediating interaction with specific protein substrates and recruitment of the kinase complex to a specific promoters. Precedent for this type of regulation comes from the finding that the HIV Tat protein binds directly to a domain within the cyclin T1 subunit of P-TEFb and thereby recruits the P-TEFb complex to early elongation complexes formed at the HIV long terminal repeat promoter (15). The Tat interaction region is located outside the cyclin box homology region of cyclin T1 (16, 17), is not present in cyclins T2 and K, and is therefore cyclin T1-specific. Since the activation of T cells is accompanied by an up-regulation of cyclin T1 levels (18, 19), it is possible that cyclin K levels may be regulated in a development or tissue specific manner to modulate the P-TEFb activity.

Work with the P-TEFb complex has shown conclusively that CDK9 is involved in transcription regulation and has led to the proposal that phosphorylation of the RNAPII CTD mediates the positive effect of P-TEFb in transcription elongation (1, 10). Here we show that CDK9-cyclin K phosphorylates the RNAPII CTD and stimulates transcription elongation but with slightly lower efficiency than the CDK9-cyclin T1 complex (Figs. 3 and 4). The transcription activity of both complexes seems to be directly proportional to their CTD kinase activity and the activity of the CDK9-cyclin K complex is lower than that of CDK9-cyclin T1 in both assays. This difference in activity might simply reflect a lower specific activity of the CDK9-cyclin K complex or might indicate that the CTD is not the preferred substrate of the CDK9-cyclin K complex. It is also entirely possible that phosphorylation of other protein factors instead of or in addition to the CTD of RNAPII might mediate CDK9-dependent transcription regulation. The identification of cyclin K as a CDK9 regulatory subunit will undoubtedly help to address many important questions that will lead to a better understanding of the mechanism by which CDK9 modulates gene expression.

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