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Regulation of CDK7 substrate specificity by MAT1 and TFIIH

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The cyclin-dependent kinase (CDK)-activating kinase CAK has been proposed to function in the control of cell cycle progression, DNA repair and RNA polymerase II (pol II) transcription. Most CAK exists as complexes of three subunits: CDK7, cyclin H (CycH) and MAT1. This tripartite CAK occurs in a free form and in association with 'core' TFIIH, which functions in both pol II transcription and DNA repair. We investigated the substrate specificities of different forms of CAK. Addition of the MAT1 subunit to recombinant bipartite CDK7–CycH switched its substrate preference to favour the pol II large subunit C-terminal domain (CTD) over CDK2. We suggest that the MAT1 protein, previously shown to function as an assembly factor for CDK7–CycH, also acts to modulate CAK substrate specificity. The substrate specificities of natural TFIIH and free CAK were also compared. TFIIH had a strong preference for the CTD over CDK2 relative to free CAK. TFIIH, but not free CAK, could efficiently hyperphosphorylate the CTD. In the context of TFIIH, the kinase also acquired specificity for the general transcription factors TFIIE and TFIIF which were not recognized by free CAK. We conclude that the substrate preference of the CDK7–CycH kinase is governed by association with both MAT1 and 'core' TFIIH.

Keywords: CDK-activating kinase (CAK)/cyclin-dependent kinase (CDK)/RNA polymerase II/TFIIH

Introduction

CAK is a cyclin-dependent kinase (CDK) which contains a catalytic CDK7 ($p40^{MO15}$) subunit, cyclin H (CycH) and the ring finger protein MAT1 (Nigg, 1996). It activates other CDKs *in vitro* by phosphorylating a conserved threonine residue in their T-loop domain (Morgan, 1995). Recently, the *in vivo* significance of CDK phosphorylation by CAK has been questioned since the enzyme with this function in budding yeast is not homologous to CAK (Kaldis *et al.*, 1996; Thuret *et al.*, 1996). CAK plays an important role in controlling transcription by phosphorylating the C-terminal domain (CTD) of the RNA polymerase II (pol II) large subunit and has been implicated in repair of UV-damaged DNA (Adamczewski *et al.*, 1996; Hoeijmakers *et al.*, 1996). The yeast CDK7 homologue, Kin28, is essential, and its conditional inactivation blocks pol II transcription and inhibits CTD phosphorylation *in vivo* (Cismowski *et al.*, 1995; Valay *et al.*, 1995; Akhtar *et al.*, 1996).

CAK has been isolated from *Xenopus* and mammalian cell extracts as a tripartite complex CDK7–CycH–MAT1, and a less abundant bipartite complex CDK7–CycH (Poon *et al.*, 1993; Devault *et al.*, 1995; Fisher *et al.*, 1995). It remains an open question whether the bipartite complex is a degradation product, although it can be reconstituted *in vitro* from recombinant subunits (Fisher *et al.*, 1995). CAK also exists as a large complex corresponding to the general transcription factor (GTF) TFIIH (Roy *et al.*, 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995; Adamczewski *et al.*, 1996). 'Holo' TFIIH can be dissociated into two subcomplexes; tripartite CAK and 'core' TFIIH, which contains at least five subunits: p62, p34, p44, ERCC2 and ERCC3 (Hoeijmakers *et al.*, 1996). An additional complex of ERCC2–CAK has also been resolved from 'holo' TFIIH (Drapkin *et al.*, 1996; Reardon *et al.*, 1996).

Reconstitution of bipartite or tripartite CAK in reticulocyte lysates or insect cells infected with recombinant baculoviruses demonstrated that the MAT1 subunit stabilizes the interaction between CDK7 and CycH (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995). The stability of bipartite human CAK is dependent on phosphorylation of Thr170 of CDK7, but this modification is not required for stability in the presence of MAT1 (Devault *et al.*, 1995; Fisher *et al.*, 1995). Based on these data, MAT1 was proposed to function as an assembly factor which stabilizes the enzyme.

The factors which affect substrate recognition by CAK have not been explored extensively; however, it has been shown that CDK phosphorylation is modulated by proteins bound to the substrate. For example, the presence of cyclins A and B enhanced the phosphorylation of CDC2 by CAK (Fisher and Morgan, 1994), whereas CycD inhibited phosphorylation of CDK2 (Higashi *et al.*, 1996). In addition, the association of CDK inhibitors with CDK–cyclin complexes inhibited their phosphorylation by CAK *in vivo* (Kato *et al.*, 1994) and *in vitro* (Aprelikova *et al.*, 1995). TFIIH phosphorylates CDC2, CDK2, TFIIF, TFIIE, TATA-binding protein (TBP) and the CTD (Lu *et al.*, 1992; Serizawa *et al.*, 1992; Ohkuma and Roeder, 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995). It is not known how efficiently the GTFs TFIIE, TFIIF and TBP are phosphorylated by free CAK. In one report, phosphorylation of a CTD peptide by reconstituted CAK was enhanced by MAT1 (Adamczewski *et al.*, 1996); however, it was not clear whether this effect was due to altered substrate specificity or simply to stabilization of the CDK7–CycH complex.

Here we have studied different forms of natural CAK immunoprecipitated from cell extracts and recombinant CAK prepared from baculovirus-infected insect cells. Our

results show that association with MAT1 and 'core' TFIIH modulates the activity of CDK7 at the level of substrate specificity.

Results

MAT1 alters the substrate specificity of recombinant CAK

The MAT1 subunit of CAK stabilizes the association of CDK7 with CycH and was proposed to serve as an assembly factor (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995). We investigated whether MAT1 might also affect the catalytic properties of CDK7. Human CAK subunits were co-expressed in Sf9 cells and immunoprecipitated by anti-CDK7 antibodies. Extensive precautions were taken to inhibit phosphatases during preparation of the recombinant proteins (see Materials and methods) as phosphorylation is important for stability of the bipartite CDK7–CycH complex (Fisher *et al.*, 1995). We found that immunoprecipitation was more effective than conventional chromatography (Ni^{2+}–NTA and S-Sepharose) in separating recombinant CAK from the endogenous CTD kinase activity in Sf9 lysates. Co-immunoprecipitation of CycH and MAT1 with CDK7 was confirmed by Western blot analysis (data not shown). We did not succeed in reconstituting an immunoprecipitable ERCC2–CAK complex by mixing extract from Sf9 cells expressing ERCC2 with extract from cells co-expressing CDK7 + CycH + MAT1 or by co-expressing all four subunits in Sf9 cells (data not shown).

In order to measure substrate preference directly, kinase reactions were performed with a mixture of two substrates: GST–CTD and GST–CDK2(K33R). The GST–CDK2-(K33R) substrate is inactive as a kinase due to a mutation in its catalytic centre. It is phosphorylated on residue T160 of CDK2 to equal extents in the presence or absence of CycA (Poon *et al.*, 1993). The GST–CTD substrate contains full-length mouse CTD with 52 heptad repeats (consensus YSPTSPS). In its hyperphosphorylated state (CTD-0), it migrates more slowly than in its hypophosphorylated CTD-A state. Kinase reactions were linear for at least 2 h, and phosphorylation of the two substrates was measured after 30 min. Under these conditions, the relative phosphorylation rates for the two substrates are a measure of substrate specificity, i.e. the ratio of k_{cat}/K_M for the two substrates. Note that this ratio is independent of substrate concentration (Fersht, 1985).

CDK7 + CycH or CDK7 + CycH + MAT1 were co-expressed in Sf9 cells. Western blotting showed equal levels of CDK7 and CycH respectively in the two lysates (data not shown). Immunoprecipitates of both lysates with anti-CDK7 antibody had significant kinase activity towards GST–CTD and GST–CDK2(K33R). This activity was dependent on infection with both CDK7 and CycH viruses (Figure 1B, lanes 1 and 2 and data not shown) and was abolished by blocking the anti-CDK7 antibody with antigenic peptide (Figure 1B, lanes 4 and 6). Tripartite CAK consistently phosphorylated GST–CTD more extensively than the bipartite complex relative to GST–CDK2 (Figure 1B, lanes 3 and 5). The average ratio of CTD to CDK2 signals was 4.06 (n = 11) for CDK7–CycH–MAT1 and 0.88 (n = 11) for CDK7–CycH co-expressed in Sf9 cells (see Figure 6). The CTD/CDK2 phosphorylation

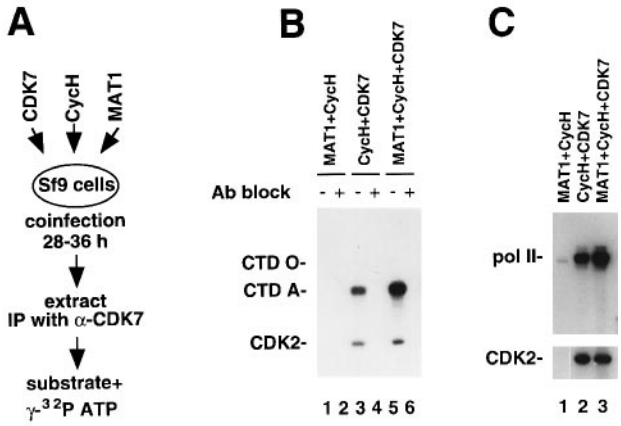

Fig. 1. MAT1 stimulates phosphorylation of RNA pol II CTD by recombinant CDK7–CycH kinase. (**A**) Scheme for preparation of recombinant CAK by co-infection of Sf9 cells. IP; immunoprecipitation. (**B**) Autoradiograph of kinase reactions with bipartite and tripartite recombinant CAK. + indicates blocking of anti-CDK7 antibody with excess antigenic peptide. The positions of phosphorylated GST–CDK2(K33R), GST–CTD-A and GST–CTD-0 are indicated. (**C**) MAT1 + CycH, CycH + CDK7 and MAT1 + CycH + CDK7 samples were prepared as in (A). Parallel kinase assays were performed with pol II and GST–CDK2(K33R).

ratios for the CDK7–CycH complex varied between 0.23 and 2.4 in different co-infections, whereas the ratio for MAT1–CDK7–CycH complexes varied between 2.9 and 5.4. We do not understand the basis for this variation; however, we always found that in parallel infections the tripartite complex had a higher CTD/CDK2 phosphorylation ratio by a factor of 4.5 on average. Side-by-side kinase reactions with immunopurified calf thymus RNA pol II (Thompson *et al.*, 1990) as substrate showed that phosphorylation by CDK7–CycH–MAT1 was also enhanced relative to CDK7–CycH by 2.2-fold (Figure 1C, lanes 2 and 3). The smaller effect observed with this substrate may result from the fact that it is purified as a mixture of hypo- and hyperphosphorylated forms.

We considered the possibility that the higher CTD/CDK2 phosphorylation ratio in the presence of MAT1 could be a non-specific effect of stabilizing CDK7–CycH which results in greater kinase activity per reaction. To test this possibility, we titrated tripartite (Figure 2A, lanes 1–3) and bipartite (Figure 2A, lanes 4–6) CAK while keeping the substrates constant. This experiment demonstrated that the enhancement of CTD versus CDK2 phosphorylation by MAT1 was independent of the total amount of kinase activity in the reaction.

To confirm the effect of MAT1 on substrate preference, we prepared CAK complexes by a different method. CDK7, CycH and MAT1 were expressed individually in Sf9 cells. Equal amounts of CDK7 and CycH extracts plus either mock-infected or MAT1-infected extract were mixed for 1 h and then immunoprecipitated with anti-CDK7 antibodies. The CAK complexes prepared in this way had approximately equal activity toward GST–CDK2(K33R) but the MAT1-containing complex had much higher activity toward GST–CTD (Figure 2B, lanes 2 and 3). We consistently observed that the bipartite complex assembled from individually expressed subunits

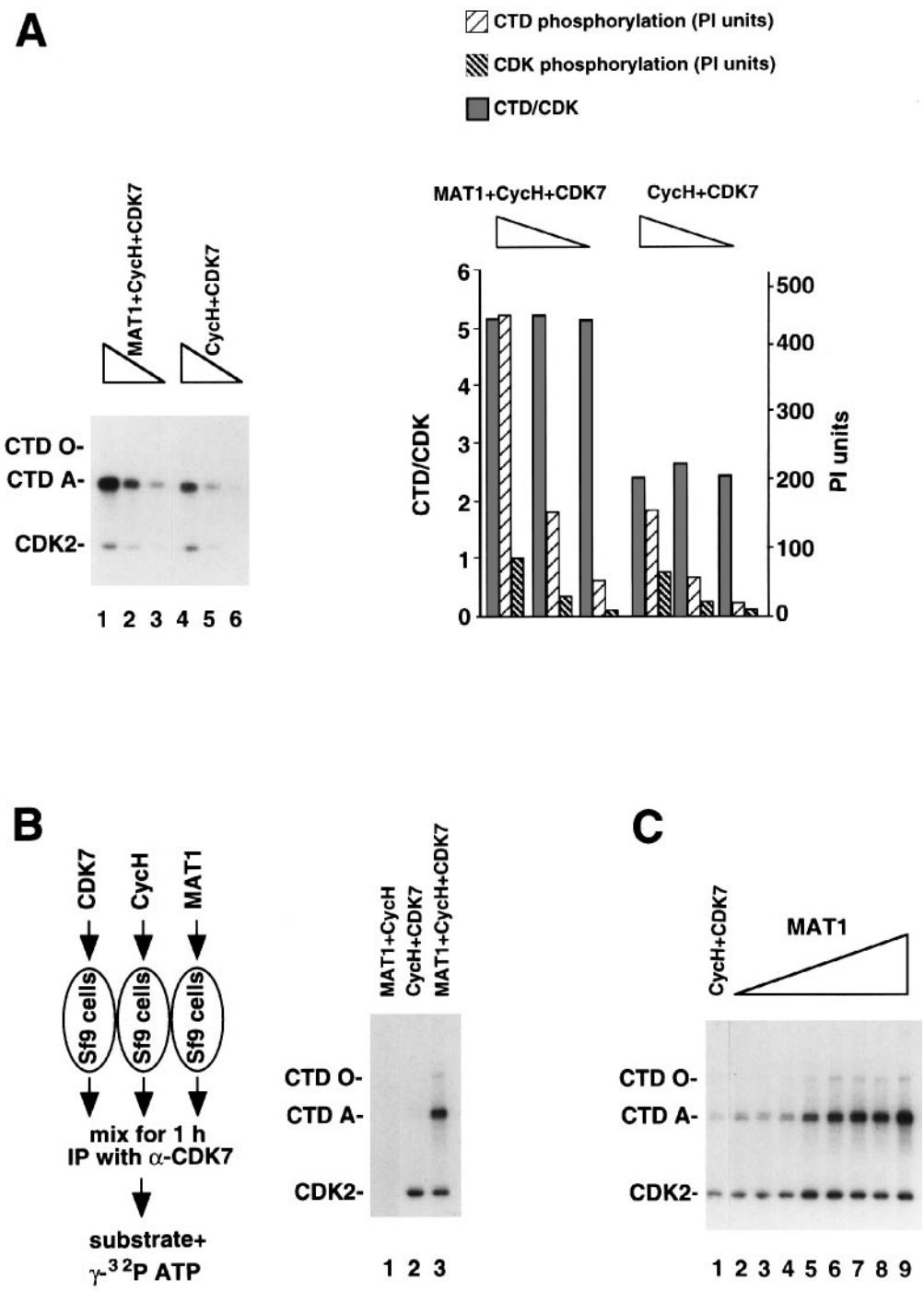

Fig. 2. MAT1 shifts CAK substrate preference. (**A**) CAK substrate preference is independent of total kinase activity. Kinase assays with baculoviral CAK complexes were as in Figure 1B. Serial 1:3 dilutions were used in lanes 1–3 and 4–6. The gel was quantified by Phosphorimager (PI) (Molecular Dynamics) and arbitrary units were plotted as well as CTD/CDK2 ratios. (**B**) Substrate preferences of bipartite and tripartite CAK reconstituted from individually expressed subunits. Extracts from 10^5 infected Sf9 cells were mixed for 1 h on ice, immunoprecipitated and assayed for kinase activity. Extract from 10^5 mock-infected Sf9 cells was added to samples 1 and 2. (**C**) Dose-dependent shift in CAK substrate specificity by MAT1. Sf9 cells were infected with CycH and CDK7 viruses at an m.o.i. of 3–5 (lanes 1–9) and MAT1 virus at an m.o.i. from 0.5 (lane 2) to 18 (lane 9). Kinase assays were performed as in Figure 1B.

had especially low reactivity toward the CTD. A control mix of MAT1- and CycH-infected extracts had no immunoprecipitable kinase activity (Figure 2B, lane 1). In summary, the substrate preferences of CAK complexes assembled by mixing of individual components behaved in the same way as those made by co-expression.

We also tested whether the effect of MAT1 on the substrate specificity of CDK7–CycH was dose dependent. Sf9 cells were infected with the CDK7 and CycH viruses at a constant m.o.i. of 3–5 and the MAT1 virus was titrated from m.o.i. 0 to 18. CAK was precipitated from each infected cell extract with anti-CDK7 antibody and assayed

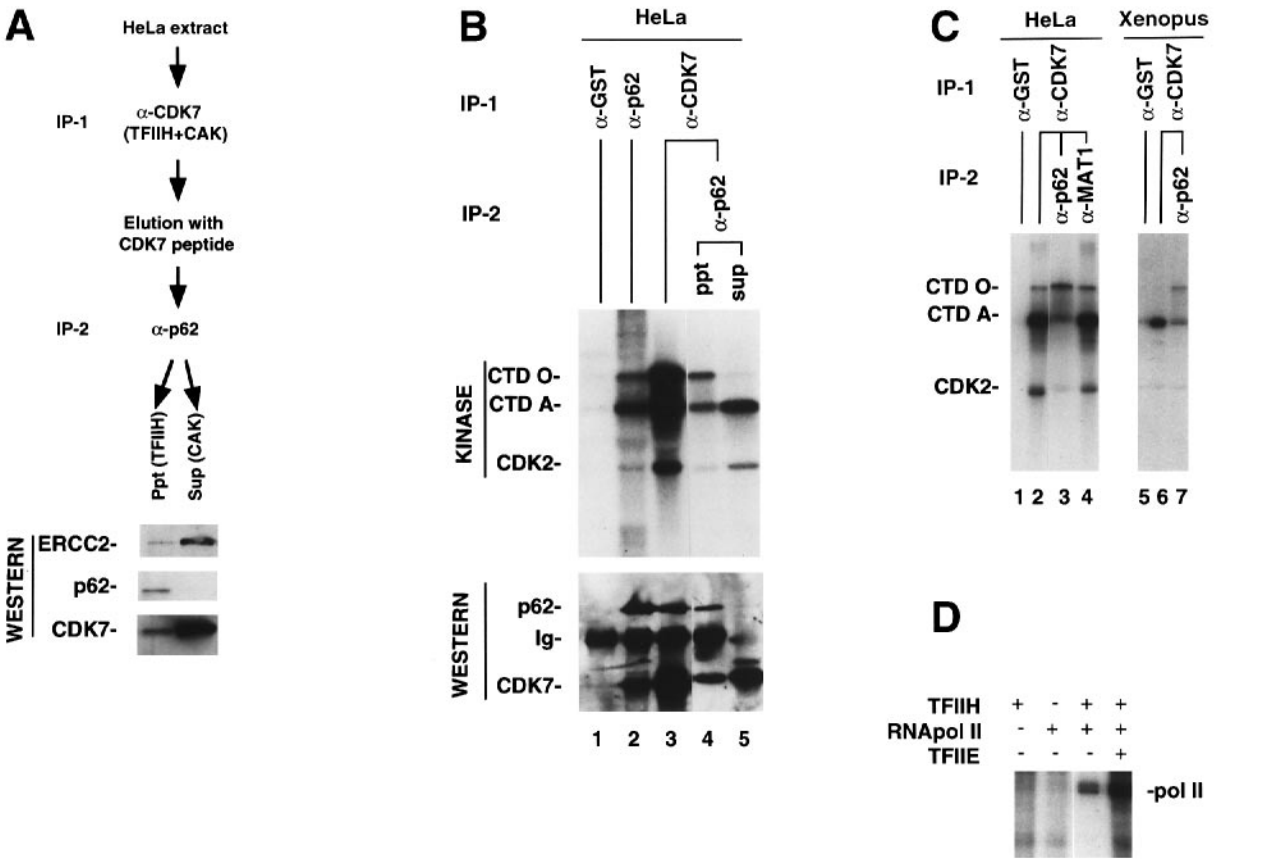

Fig. 3. Natural free CAK and TFIIH differ in their substrate specificities. (**A**) Scheme for preparation of TFIIH and free CAK from HeLa cell extract by sequential immunoprecipitation (IP) with rabbit anti-CDK7 and anti-p62 peptide antibodies. The final TFIIH and free CAK preparations were analysed by Western blotting with monoclonal anti-p62 (3C9), anti-CDK7(2F8) and anti-ERCC2 (2F6). A 2-fold greater fraction of the TFIIH was loaded relative to free CAK. (**B**) Kinase and Western blot analysis of HeLa TFIIH (lane 4) and free CAK (lane 5) prepared as in (A). Lanes 1–3: controls immunoprecipitated with anti-GST, anti-p62 or anti-CDK7 with 2-fold greater loading of lane 2. (**C**) Comparison of the kinase activities of TFIIH with total CAK from HeLa cells and *Xenopus* oocytes. TFIIH (lanes 3 and 7) and total CAK (lanes 2, 4 and 6) were prepared by single or double IP as indicated; 2.5-fold larger fractions of TFIIH (lanes 3 and 7) were analysed relative to total CAK (lanes 2 and 6). (**D**) Immunopurified HeLa TFIIH is activated by TFIIE. TFIIH was purified as in (A) and kinase activity toward RNA pol II was assayed as in Figure 1C. Recombinant TFIIE was added at 5 μg /ml.

with GST–CTD and GST–CDK2(K33R) substrates (Figure 2C). This experiment showed that enhancement of the CTD phosphorylation relative to CDK2 phosphorylation was approximately linearly related to the amount of MAT1 virus used to infect the culture. We conclude that the MAT1 subunit confers on CDK7–CycH a preference for RNA pol II CTD as a substrate over CDK2.

TFIIH and free CAK differ in their substrate preference

To compare the substrate specificities of naturally occurring isoforms of CAK, we purified them by immunoprecipitation from HeLa whole cell extract according to the scheme in Figure 3A. Free CAK and TFIIH-associated CAK were immunoprecipitated with anti-CDK7 peptide antibody, and then eluted from the precipitate with excess peptide. The eluted material was re-immunoprecipitated with antibodies against either MAT1 or the p62 subunit of TFIIH. The anti-p62 precipitate contains TFIIH while the supernatant contains free CAK. The anti-MAT1 precipitate is a mixture of TFIIH and free CAK and serves as a control for total CAK activity after the second round of immunoprecipitation. This purification strategy has the advantage that both the TFIIH-associated and the free form of CAK were treated identically before the kinase assay. Western blotting showed that the anti-p62 precipitate contained both p62 and CDK7, whereas the supernatant was effectively cleared of 'core' TFIIH as demonstrated by the absence of p62 (Figures 3A and B and 5). The presence of ERCC2 in the supernatant from the anti-p62 immunoprecipitation indicates that our 'free' CAK preparation also contains ERCC2–CAK (Figure 3A). Attempts to purify bipartite CAK from anti-CDK7 eluates by depletion with anti-MAT1 antibodies were unsuccessful, probably because of the low abundance or instability of this complex in HeLa cells (Fisher *et al.*, 1995).

Free CAK and TFIIH immunopurified from HeLa cells both had abundant kinase activity whereas anti-GST control immunoprecipitates had none (Figure 3B, lane 1; C, lanes 1 and 5). There were substantial differences between the kinase activities of the CAK and TFIIH samples. First, TFIIH had a stronger preference for the GST–CTD substrate over GST–CDK2(K33R) than did free CAK (Figure 3B, lanes 4 and 5). The average ratios of total CTD/CDK2 phosphorylation signals were 14.4 and 4.7 for TFIIH ($n = 7$) and CAK ($n = 5$), respectively (see also Figure 6). Secondly, the TFIIH-associated kinase caused a pronounced mobility shift of a fraction of the

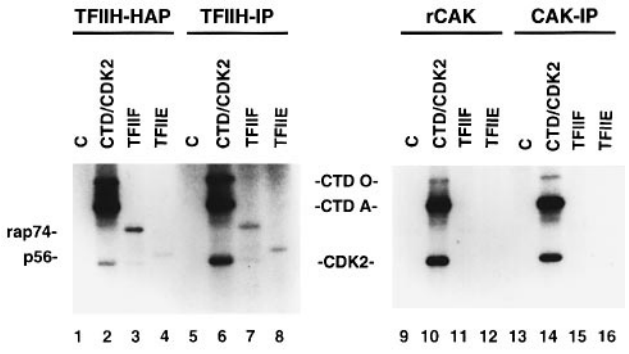

Fig. 4. TFIIH but not CAK can phosphorylate TFIIE and TFIIF. Kinase reactions were performed with: lanes 1–4, conventionally purified TFIIH (HAP fraction) (Gérard *et al.*, 1991); lanes 5–8, immunoprecipitated TFIIH (as in Figure 3A); lanes 9–12, soluble recombinant baculoviral tripartite CAK, prepared as in Figure 1A and eluted with antigenic peptide; lanes 13–16, soluble HeLa CAK prepared as in Figure 3A. Controls, C, contained no substrate. The positions of phosphorylated GST–CDK2(K33R), GST–CTD-A, GST–CTD-0, the rap74 subunit of TFIIF and the p56 subunit of TFIIE are indicated. The TFIIF preparation contains an ~50 kDa degradation product of rap74 which is also phosphorylated.

GST–CTD substrate to the slowly migrating hyperphosphorylated CTD-0 form (Figure 3A, lane 4). In contrast, free CAK did not convert GST–CTD to the CTD-0 form (Figure 3B, lane 5). Note that total CTD phosphorylation (CTD-0 + CTD-A) by TFIIH (Figure 3B, lane 4) was approximately equal to that by free CAK (Figure 3B, lane 5) although there was substantially less CDK7 in the TFIIH sample as determined by Western blotting. The average ratios of CTD-0/CTD-A signals were 1.12 and 0.04 for TFIIH ($n = 7$) and free CAK ($n = 5$), respectively (see also Figure 6). The accumulation of GST–CTD molecules in the CTD-0 position when phosphorylated by TFIIH (Figure 3B and C) was not an effect of a limiting amount of the GST–CTD substrate since most of it remained at the CTD-A position as detected by Coomassie staining (data not shown). TFIIH prepared by an alternative protocol (anti-p62 immunoprecipitation followed by peptide elution and re-precipitation with anti-CDK7) also had kinase activity, which extensively converted GST–CTD to the CTD-0 form (data not shown) as did conventionally purified TFIIH (Figure 4, lane 2).

We considered the possibility that free CAK may be unable to hyperphosphorylate the CTD as a result of a non-specific inhibitor in the anti-CDK7 eluate which is removed in a second immunoprecipitation step. To control for such an artefact, we immunoprecipitated the anti-CDK7 eluate with anti-MAT1 in parallel with anti-p62 (Figure 3C). The anti-MAT1 precipitate, which contained free CAK plus TFIIH, phosphorylated GST–CTD and GST–CDK2(K33R) similarly to the original anti-CDK7 eluate. As expected, the anti-p62 immunoprecipitate containing only TFIIH preferentially phosphorylated the CTD over CDK2, and hyperphosphorylated a significant fraction of the CTD. This result therefore confirmed the enhanced CTD/CDK2 phosphorylation ratios for TFIIH relative to free CAK.

We also compared the abilities of total CAK and TFIIH purified from *Xenopus* oocytes to hyperphosphorylate the CTD. Total CAK was prepared by immunoprecipitation with anti-*Xenopus* CDK7 peptide antibody and TFIIH was prepared by CDK7 peptide elution and re-precipitation with cross-reactive anti-p62 antibody (Figure 3C, lanes 5–7). As we observed for the human enzymes, the ability to hyperphosphorylate the CTD co-purified with TFIIH (Figure 3C, compare lanes 6 and 7). In conclusion, the experiments in Figure 3 show that the ability to hyperphosphorylate the CTD is greatly enhanced in TFIIH relative to free CAK.

The increased phosphorylation of CTD by TFIIH relative to free CAK could result from contamination of immunoprecipitated TFIIH with TFIIE which stimulates the TFIIH kinase (Lu *et al.*, 1992). Two lines of evidence argue against this possibility. First, in Western blots, we did not detect either the p34 or the p56 subunit of TFIIE in immunoprecipitates with anti-CDK7, anti-p62 or anti-MAT1 antibodies (data not shown). Secondly, we tested whether the kinase activity of immunopurified TFIIH was still responsive to addition of TFIIE (Figure 3D). In this experiment, TFIIH purified by sequential precipitation with anti-CDK7 and anti-p62 antibodies was incubated with calf thymus pol II in the presence or absence of recombinant TFIIE. The kinase activity of immunopurified TFIIH was activated 5-fold by TFIIE, in agreement with the level of activation of conventionally purified TFIIH by TFIIE (Lu *et al.*, 1992; Yankulov *et al.*, 1995). We conclude that TFIIE contamination is not responsible for the enhanced CTD phosphorylation by TFIIH relative to free CAK.

TFIIE and TFIIF are phosphorylated by TFIIH but not by free CAK

In addition to the CTD, TFIIH phosphorylates the rap74 subunit of TFIIF, the p56 subunit of TFIIE, and TBP (Ohkuma and Roeder, 1994). The ability of free CAK to phosphorylate these substrates has not been investigated. We compared phosphorylation of these GTFs by TFIIH and both natural and recombinant free CAK. To control for artefacts resulting from a contaminating kinase or immobilization of our immunoprecipitated TFIIH, we also tested the kinase activity of highly purified soluble TFIIH (HAP fraction TFIIH; Gérard *et al.*, 1991). Parallel kinase assays were carried out with recombinant TFIIF, recombinant TFIIE and a mix of GST–CTD and GST–CDK2(K33R) as substrates. As expected, both TFIIH preparations phosphorylated the CTD more efficiently than CDK2 and hyperphosphorylated a significant amount of the CTD substrate. The conventionally purified TFIIH had a 2.5-fold higher CTD/CDK2 phosphorylation ratio than immunoprecipitated TFIIH (Figure 4, lanes 2 and 6). Immunoprecipitated TFIIH and conventionally purified TFIIH phosphorylated the TFIIE p56 subunit and the TFIIF rap74 subunit (Figure 4, lanes 3, 4, 7 and 8) whereas neither recombinant nor HeLa-derived CAK phosphorylated either of these substrates to a detectable level (Figure 4, lanes 11, 12, 15 and 16). We consistently observed that phosphorylation of TFIIF by TFIIH was more efficient than by TFIIE. In an experiment not shown, CAK also failed to phosphorylate TBP. These results indicate that free CAK does not recognize TFIIE and TFIIF as substrates; however, it acquires specificity for these substrates upon association with TFIIH.

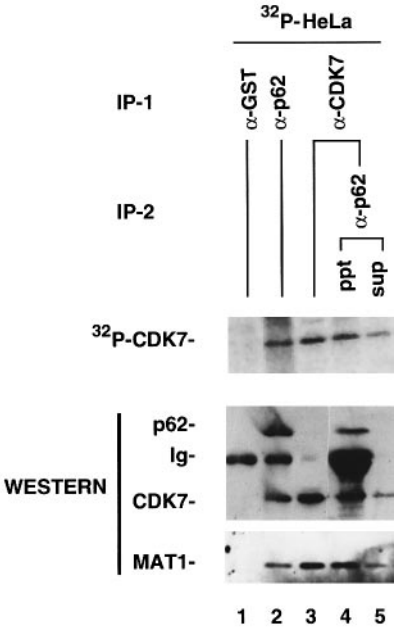

Fig. 5. CDK7 is phosphorylated *in vivo* equally in free CAK and TFIIH. HeLa cells were metabolically labelled with $^{32}PO_4$, and TFIIH (lane 4) and free CAK (lane 5) were prepared by double IP as in Figure 3A. As controls, single IPs with anti-GST, anti-p62 and anti-CDK7 are shown in lanes 1–3. Samples were transferred to PVDF membrane and [^{32}P]CDK7 was detected by Phosphorimager. Subsequently, Western blot analysis was performed with monoclonal anti-p62 (3C9), anti-CDK7 (2F8) and rabbit anti-MAT1 antibodies.

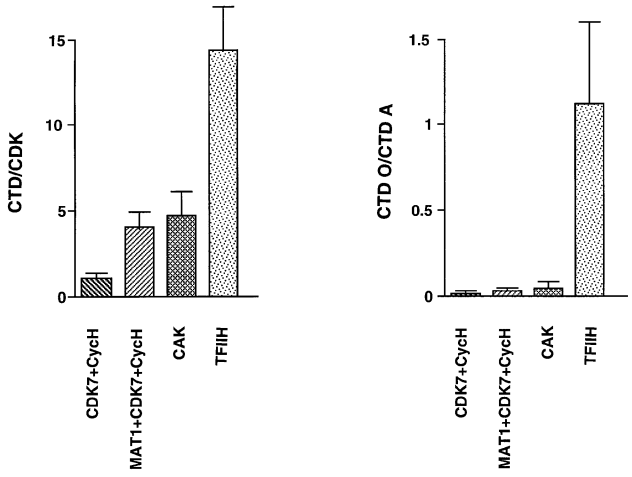

Fig. 6. Substrate specificities of different CAK complexes. CycH–CDK7 and MAT1–CycH–CDK7 values are averages from 11 experiments with baculoviral complexes reconstituted by co-infection of Sf9 cells. CAK and TFIIH indicate average ratios from five and seven experiments, respectively, with free CAK and TFIIH prepared from HeLa cells as described in Figure 3A. The CTD value is the sum of CTD-0 + CTD-A. CTD/CDK2 and CTD-0/CTD-A values represent ratios of Phosphorimager values.

Phosphorylation of CDK7 in TFIIH and free CAK

Phosphorylation of CDK7 on residue T170 is required for formation of a stable bipartite complex with CycH but is not essential in the presence of MAT1 (Devault *et al.*, 1995; Fisher *et al.*, 1995). We tested the hypothesis that a difference in phosphorylation of CDK7 in TFIIH compared with free CAK contributes to the difference in substrate specificity between these two forms of the enzyme. HeLa cells were ^{32}P-labelled and TFIIH and free CAK were purified by sequential immunoprecipitation with anti-CDK7 and anti-p62 antibodies (see Figure 3A). Samples were resolved on an SDS-gel, transferred to PVDF membrane and $^{32}PO_4$ incorporation into CDK7 was quantified by Phosphorimager. The membrane was then reacted with anti-CDK7, anti-p62 and anti-MAT1 antibodies. The anti-p62 Western blot showed that the free CAK (Figure 5, lane 5) was not substantially contaminated with TFIIH (Figure 5, lane 4). Comparison of the ^{32}P and Western blot signals did not reveal a significant difference in relative phosphate incorporation into the CDK7 subunits of TFIIH versus free CAK. These results, therefore, do not indicate that a change in the overall level of CDK7 phosphorylation causes the difference in substrate specificity between TFIIH and free CAK.

Discussion

CAK is regulated at the level of substrate specificity

CAK exists in at least two distinct states in the cell; as a free complex and as a component of the pol II general transcription factor TFIIH. CAK phosphorylates many substrates *in vitro*, including several CDKs, the GTFs TFIIE, TFIIF and TBP, and the CTD of the largest subunit of RNA pol II (Morgan, 1995; Hoeijmakers *et al.*, 1996; Nigg, 1996). This broad range of targets could indicate that the enzyme has a relaxed substrate specificity, at least *in vitro*. Alternatively, the different isoforms of CAK could be restricted in their specificity to different substrates. In order to investigate this question, various forms of recombinant and natural CAK were prepared and assayed with mixtures of substrates to determine their substrate preferences.

We observed that phosphorylation of RNA pol II and a full-length CTD fusion protein by recombinant CAK reconstituted in insect cells is significantly enhanced by MAT1 (Figures 1 and 2). The CTD/CDK2 phosphorylation ratio increased from 0.88 (n = 11) for recombinant CDK7–CycH to 4.06 (n = 11) for CDK7–CycH–MAT1. Our data did not reveal a significant effect of MAT1 on phosphorylation of GST–CDK2, but we cannot exclude the possibility of an effect in the presence of partner cyclins. The catalytic activity of recombinant tripartite CAK closely resembled that of free cellular CAK in the ratio of both CTD/CDK2 and CTD-0/CTD-A phosphorylation (see Figure 6). We think it unlikely, therefore, that MAT1 enhanced CTD phosphorylation by recruiting insect 'core' TFIIH to the human CDK7–CycH complex. This possibility is also unlikely because TFIIH is not an abundant factor and because MAT1 functions in a species-specific way (Devault *et al.*, 1995).

CAK phosphorylation of the CTD was stimulated significantly by association with TFIIH. The average ratios of CTD/CDK2 phosphorylation signals were 4.06 (n = 11) and 4.67 (n = 5) for recombinant tripartite CAK and free cellular CAK, respectively, whereas the ratio for TFIIH was 14.4 (n = 7) (see Figure 6). Furthermore, a far higher proportion of the CTD substrate was hyperphosphorylated by TFIIH than by free CAK (Figures 3 and 4). The average ratio of CTD-0/CTD-A signals was 15-fold higher for TFIIH than for free CAK (Figure 6).

Hence, our data imply that TFIIH phosphorylates many serine residues each time it binds a substrate molecule, whereas free CAK phosphorylates only one or a few residues.

Neither recombinant tripartite CAK nor free cellular CAK phosphorylated TFIIE (p56) or TFIIF (rap74) (Figure 4). In contrast, conventionally purified and immunoprecipitated TFIIH phosphorylated both these substrates (Figure 4) (Ohkuma and Roeder, 1994; Yankulov *et al.*, 1995). This observation suggests that the ability to phosphorylate TFIIE and TFIIF is acquired by CAK upon association with 'core' TFIIH.

Previously, a monoclonal anti-CDK7 antibody was used to immunoprecipitate RNA pol II holoenzyme containing all the GTFs (Ossipow *et al.*, 1995). Under our immunoprecipitation conditions, we did not detect RNA pol II or TFIIE in the anti-CDK7 or anti-p62 precipitates by Western blotting (data not shown). In addition, TFIIH which had been purified extensively over seven columns (Gérard *et al.*, 1991) phosphorylated TFIIF and TFIIE and hyperphosphorylated CTD similarly to the immunoprecipitated TFIIH (Figure 4). Hence, we believe that these are properties of TFIIH *per se* rather than of a higher order complex of transcription factors or a contaminating kinase.

Recently, a complex containing CAK (CDK7–CycH–MAT1) and ERCC2 was separated from TFIIH (Drapkin *et al.*, 1996; Reardon *et al.*, 1996). Consistent with these observations, we found appreciable amounts of ERCC2 but no p62 in our free CAK preparations (Figure 3A and B). The kinase activity of free CAK containing ERCC2 toward CDK2 and CTD substrates closely resembled that of recombinant tripartite CAK rather than TFIIH (see Figure 6). In addition, free CAK did not phosphorylate TFIIE or TFIIF (Figure 4). These data argue that association of CAK with ERCC2 is not sufficient to confer the characteristic substrate specificity of the TFIIH kinase.

In summary, we suggest that the MAT1 subunit of CAK, previously shown to facilitate assembly of CDK7 with CycH, also shifts the enzyme's substrate preference in favour of the CTD; and that a high stoichiometry of CTD phosphorylation requires 'core' TFIIH.

Mechanisms of CDK7 substrate specificity regulation

Modulating the conformation of the catalytic site may be important in control of protein kinase activity (Morgan and De Bondt, 1994). For example, the extensive conformational changes which occur when the conserved Thr160 becomes phosphorylated (Russo *et al.*, 1996) might affect substrate recognition in some CDKs. However, our experiments revealed no evidence for involvement of phosphorylation in modulating CDK7 substrate specificity. There was no correlation between altered substrate recognition by CDK7 in free CAK and TFIIH and altered overall phosphorylation of the enzyme (Figure 5). This experiment does not exclude the possibility that CDK7 is phosphorylated to equal extents on different residues in different complexes. Addition of MAT1 to CycH–CDK7 dramatically alters its behaviour in gel filtration experiments, suggesting a significant conformational change (Fisher *et al.*, 1995). Such an effect of MAT1 could be involved both in promoting binding to CycH and in phosphorylation of the CTD.

CTD hyperphosphorylation is greatly enhanced in TFIIH-associated CAK relative to free CAK (see Figure 6). GST–CTD hyperphosphorylation by free CAK was observed previously when the enzyme was immunoprecipitated with antibodies raised against GST–MAT1 (Tassan *et al.*, 1995). In this case, cross-reaction of the antibody with the GST–CTD substrate presumably stabilized the kinase–substrate interaction and promoted processive phosphorylation (Tassan *et al.*, 1995). An interaction between 'core' TFIIH and the CTD may act in a similar manner to enhance hyperphosphorylation. Such an interaction could, for example, decrease the dissociation rate of the substrate thereby allowing multiple phosphorylations on a single GST–CTD molecule. Interactions between TFIIE and TFIIF with 'core' TFIIH (Maxon *et al.*, 1994) may be responsible for the specificity of TFIIH-associated CAK for these substrates.

Implications for the regulation of the activity of other CDKs

Here we demonstrate that the substrate preference of CAK is modulated by its association with MAT1 and TFIIH. The control of other CDKs at the level of substrate specificity has not been explored extensively. It has been observed, however, that CycA–CDC2 and CycA–CDK2 kinases phosphorylate the Rb-related protein p107, whereas the corresponding CycB complexes do not (Peeper *et al.*, 1993). In addition, interaction of p107 with CycA–CDK2 or CycE–CDK2 apparently restricted the substrate specificity of these kinases to p107 and not pRB (Zhu *et al.*, 1995). The mechanism of regulating kinase activity by modulation of substrate specificity may, therefore, be of significance to other members of the CDK family in addition to CAK.

Materials and methods

Antibodies

Affinity-purified rabbit anti-peptide antibodies were prepared against human CDK7 residues 329–343 (KRTEALEQGGLPKK), human MAT1 residues 131–148 (DVIQKNKLKLTREQEELE), human CycH C-terminus (SKKSKHEEEEWTDDDLVESL) and human TFIIH p62 C-terminus (YNKLHTWQSRRLMKKT). The anti-p62 antibody cross-reacted with the *Xenopus laevis* homologue in Western blots (data not shown). Affinity-purified anti-*X.laevis* CDK7 C-terminal peptide antibody (CDQKDIAKKLSF) was purchased from Upstate Biotechnology Inc. Rabbit anti-GST antibodies were affinity purified. Monoclonal antibodies against human p62 (3C9), (Fischer *et al.*, 1992), CDK7 (2F8) (Roy *et al.*, 1994) and ERCC2 (2F6) (Schaeffer *et al.*, 1994) were gifts of Dr J.-M.Egly. Affinity-purified rabbit polyclonal anti-TFIIE antibody raised against a complex of bacterially expressed p56-His_6p34 was provided by M.Pandes.

Protein kinase assay

Kinase buffer [50 mM KCl, 20 mM Tris–HCl pH 8.0, 7.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 5 mM 2-glycerophosphate, 1 μM microcystin, 100 μg/ml bovine serum albumin (BSA), 10 μM ATP, 2–10 μCi [γ-^{32}P]ATP per reaction; 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 μg/ml pepstatin] was pre-mixed on ice with protein substrates and added to 0.5–3 μl of kinase in soluble form or immunoprecipitated on protein A–Sepharose beads in a final volume of 20 μl. Reactions were incubated for 30 min at 30°C and terminated by addition of 5 μl of 5× SDS loading buffer. Aliquots of the kinase reactions were resolved on 7.5% SDS gels which were fixed and stained with Coomassie Brilliant Blue to verify equal loading. Dried gels were quantified by PhosphorImager (Molecular Dynamics).

The following protein substrates were used: a mix of 50 μg/ml each of GST–CTD (Peterson *et al.*, 1992) and GST–CDK2(K33R) (Poon

et al., 1993); 60 μg/ml recombinant TFIIE and TFIIF; and 10 μg/ml RNA pol II. Expression and purification of recombinant TFIIF, TFIIE, GST–CTD and GST–CDK2(K33R) were described previously (Yankulov *et al.*, 1995). Immunoaffinity-purified calf thymus pol II (Thompson *et al.*, 1990) (a gift of Dr J.Greenblatt) contained both the hypophosphorylated (pol II A) and hyperphosphorylated (pol II 0) forms of the enzyme.

Expression and purification of CAK from insect cells

Baculoviruses, expressing human CDK7, CycH, MAT1 and ERCC2, were gifts of Drs D.Morgan, R.Fisher and D.Siderovski. Sf9 cells (7×10^5/ml) were infected at an m.o.i. of 3–5 for each virus (except in Figure 2C), harvested 28–36 h post-infection, washed twice in phosphate-buffered saline (PBS) and lysed in ice-cold IPL buffer [10 mM Na HEPES pH 7.5, 10 mM KCl, 0.4 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-glycerophosphate, 0.5 mM Na_3VO_4, 1 mM NaF, 0.2% NP-40, 1 μM microcystin, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF)]. KCl was added to 0.4 M, the homogenate was rocked for 30 min at 4°C and clarified by centrifuging for 15 min at 13 000 *g*. The supernatant was frozen at –70°C after adding glycerol to 20%. CAK activity from extract corresponding to 10^5 cells was immunoprecipitated with ~1 μg of anti-CDK7 antibodies on protein A–Sepharose beads (Pharmacia). Beads were washed four times for 10 min in IPL buffer containing 0.3 M KCl and 0.2% NP-40, washed once more in kinase buffer without ATP and BSA, and used for kinase assays. Under these conditions, the kinase reaction was linear for >2 h.

TFIIH

HeLa cells were washed twice in PBS and lysed as described for Sf9 cells, except that KCl was added to 0.35 M. The extract was diluted immediately with an equal volume of lysis buffer without KCl, clarified at 45 000 r.p.m. in the SW55 Beckman rotor for 30 min and filtered through a 0.22 μm filter. The supernatant was pre-cleared by incubation for 2 h with 1/10 vol of protein A–Sepharose beads. Between 5 and 10 μg of affinity-purified anti-peptide antibodies were used to immunoprecipitate p62- or CDK7-containing complexes. After five washes (15 min each) in IPL buffer plus 0.175 M KCl, the immunoprecipitates were eluted with a 1000-fold molar excess of the corresponding antigenic peptide for 2–18 h. The eluates were filtered through Bio-Rad spin columns to remove any beads, and immunoprecipitated with a second antibody. The resulting supernatant and beads (after three washes of 10 min) were used for kinase assays.

Highly purified TFIIH HAP fraction (Gérard *et al.*, 1991) was a gift from J.-M.Egly.

Metabolic labelling of HeLa cells

HeLa cells (5×10^7) were washed in phosphate-free media and incubated with 0.9 mCi of $^{32}PO_4$/ml for 3–4 h. Extraction was as described above except that clarification was for 15 min at 13 000 *g* and filtration was omitted.

Xenopus laevis oocyte extract was prepared as described in Yankulov *et al.* (1996) except that the extraction buffer was supplemented with 0.5 mM Na_3VO_4, 1 mM NaF and 1 μM microcystin.

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