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Phosphorylation of yeast TBP by protein kinase CK2 reduces its specific binding to DNA

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Abstract Protein kinase CK2 is a ubiquitous Ser/Thr kinase which phosphorylates a large number of proteins including several transcription factors. Recombinant *Xenopus laevis* CK2 phosphorylates both recombinant *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* TATA binding protein (TBP). The phosphorylation of TBP by CK2 reduces its binding activity to the TATA box. CK2 copurifies with the transcription factor IID (TFIID) complex from HeLa cell extracts and phosphorylates several of the TBP-associated factors within TFIID. Taken together these findings argue for a role of CK2 in the control of transcription by RNA polymerase II through the modulation of the binding activity of TBP to the TATA box.

Key words: Protein kinase CK2; TBP phosphorylation; TBP associated factor phosphorylation; Binding activity

1. Introduction

The TATA binding protein (TBP) is a component of the basic transcription factor IID (TFIID) which plays an important role in the formation of the pre-initiation transcription complex involving RNA polymerase II [1]. As its name indicates, TBP has the function of binding specifically to the TATA containing DNA sequence which is frequently upstream of most genes transcribed by RNA polymerase II and which is an important part of the promoter elements of these genes [2].

TBP, however, play other roles in transcription that do not involve the specific recognition of the TATA motif as is evident from the fact that this protein is also essential for the correct functioning of RNA polymerases I and III, whose genes do not include TATA sequences in their promoters [3]. These other roles involve protein-protein interactions with other factors that participate in the complex polymer required for transcription and its regulation.

There is much evidence that points to phosphorylation of transcription components as an important mechanism for the regulation of gene expression. Protein kinase CK2 is a ubiquitous Ser/Thr protein kinase that phosphorylates a large number of proteins, including a large number of transcription factors [4]. Several years ago, protein kinase CK2 (also called casein kinase II) was reported to phosphorylate the RNA polymerases and this phosphorylation was proposed to regulate transcriptional activity [5]. More recently, studies with RNA polymerase III from *Saccharomyces cerevisiae* indicated that CK2 phosphorylation was required for the transcriptional activity of this enzyme and that the factor phosphorylated by CK2 was the TBP subunit of factor TFIIIB [6]. Furthermore, it has recently been reported that CK2 copurifies with epitope-tagged RNA polymerase I from mammalian cell extracts [7].

This work describes in vitro experiments that confirm the capacity of vertebrate CK2 to phosphorylate the TBP from both *S. cerevisiae* and *Schizosaccharomyces pombe* but not the equivalent human recombinant factor. However, several TBP associated factors (TAFs) of the human TFIID complex are phosphorylated by CK2. Furthermore, it is demonstrated that CK2 phosphorylation of TBP from both types of yeast causes a significant decrease in its binding capacity to the TATA motif.

2. Materials and methods

2.1. Purification of recombinant protein

Recombinant *Xenopus laevis* CK2α and CK2β subunits were expressed and purified by NTA-agarose chromatography according to published procedures [8]. GST-CK2β was purified by glutathione-agarose chromatography. Recombinant *S. cerevisiae* TBP and *S. pombe* TBP were expressed and purified according to Maldonado et al. [9]. Recombinant TFIIA was expressed and purified according to Sun et al. [10]. The recombinant proteins are at least 90% pure as judged by SDS-PAGE followed by Coomassie blue staining.

2.2. Phosphorylation assays

The phosphorylation assays were performed in a 25 μl reaction assay which contained 50 mM HEPES pH 7.8, 100 mM KCl, 7 mM $MgCl_2$, 0.5 mM DTT and 100 μM [^{32}P]γATP (500–1000 cpm/pmol). Protein components were added as indicated in the figure legends and incubated for 15 min at 30°C. The reactions were stopped with 5 μl of 5×Laemmli loading buffer and separated by SDS-PAGE in a 12% acrylamide gel, dried and exposed overnight to X-ray films.

2.3. Binding assays

The gel retardation assays were performed in a 20 μl reaction volume using: 0.1–1 ng of a 3′-end-labelled DNA fragment containing the Ad-MLP TATA motif (approximately 5000 cpm) extending from −40 to +20. The reaction mixtures contained 10 mM HEPES pH 7.8, 4 mM $MgCl_2$, 4 mM ammonium sulfate, 8% (v/v) glycerol, 2% (w/v) polyethylene glycol 8000, 60 mM KCl, 5 mM β-mercaptoethanol, 0.2 mM EDTA and 100 ng of poly(dG)-poly(dC). The reactions were incubated at 30°C for 30 min. Those binding assays which contained TFIIA were analyzed on 5% acrylamide gel containing 0.5×TBE buffer (45 mM Tris-borate pH 8.0, 1 mM EDTA). The same buffer and concentration were used to run the gel. However, the binding assays performed in the absence of TFIIA were analyzed on 5% acrylamide gel containing 0.5×TBE-5 mM $MgCl_2$ and run in the same buffer. The gels were dried and the complexes visualized by autoradiography.

2.4. Western blot analysis

The proteins were separated on 10% SDS-PAGE gels and trans-

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Abbreviations: CK2, casein kinase II; CK2α and CK2β, the α and β subunits of protein kinase CK2; TBP, TATA binding protein; TAF, TBP associated factor; TFIID, transcription factor IID; TFIIA, transcription factor IIA

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ferred to Immobilon membranes (Millipore). The membranes were incubated in 10% v/v calf serum in TTBS (10 mM Tris pH 7.5, 300 mM NaCl and 0.05% v/v of Tween 20). After blocking the membranes were incubated overnight with affinity-purified antibodies at 0.5 μg/ml in 5% v/v calf serum in TTBS. After washing with TTBS, the blots were incubated for 30 min with secondary antibodies conjugated to alkaline phosphatase (Promega) at 1:7000 dilution in TTBS. The color was developed according to the manufacturer's instructions.

2.5. Affinity purification of TFIID

TFIID was purified from the 1 M phosphocellulose derived fraction of nuclear extract of HeLa cells which contained N-terminal epitope tagged TBP with a nine residue peptide sequence from influenza hemagglutinin (HA peptide). This stable cell line expresses the epitope tagged TBP which was inserted into HeLa cells using a retrovirus vector [11]. The epitope tagged TFIID complex was purified according to Maldonado et al. [12]. Briefly, the 1 M phosphocellulose derived fraction was incubated with monoclonal antibody 12CA5 covalently bound to protein A-agarose beads and washed extensively with a buffer containing 20 mM HEPES pH 7.8, 10% v/v glycerol, 1 mM DTT, 0.5 mM EDTA, 500 mM KCl, 0.5 mM PMSF and 0.05% v/v of NP-40. The bound TFIID was eluted with 2 mg/ml of the synthetic HA peptide in the same buffer. The fractions were analyzed by Western blot with monoclonal antibody 12CA5 and those fractions containing TBP were pooled, dialyzed and used for the phosphorylation assays.

3. Results

3.1. The phosphorylation of yeast TBP by CK2

Purified recombinant TBP from *S. cerevisiae* and *S. pombe*

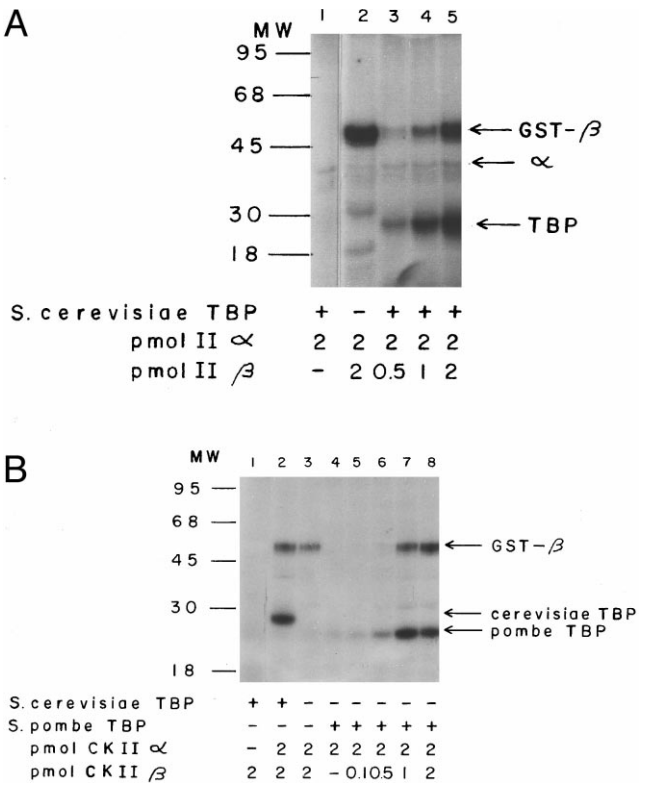

Fig. 1. Phosphorylation of recombinant *S. cerevisiae* and *S. pombe* TBP by *Xenopus laevis* CK2. Each polypeptide was incubated with different combinations of CK2α and CK2β (the latter as a fusion protein of GST-B) as indicated at the bottom of the figure. The phosphorylation reactions were performed as described in Section 2. The products were separated on 12% SDS-PAGE gels and analyzed by autoradiography. A: Phosphorylation of *S. cerevisiae* TBP. B: Phosphorylation of *S. pombe* TBP.

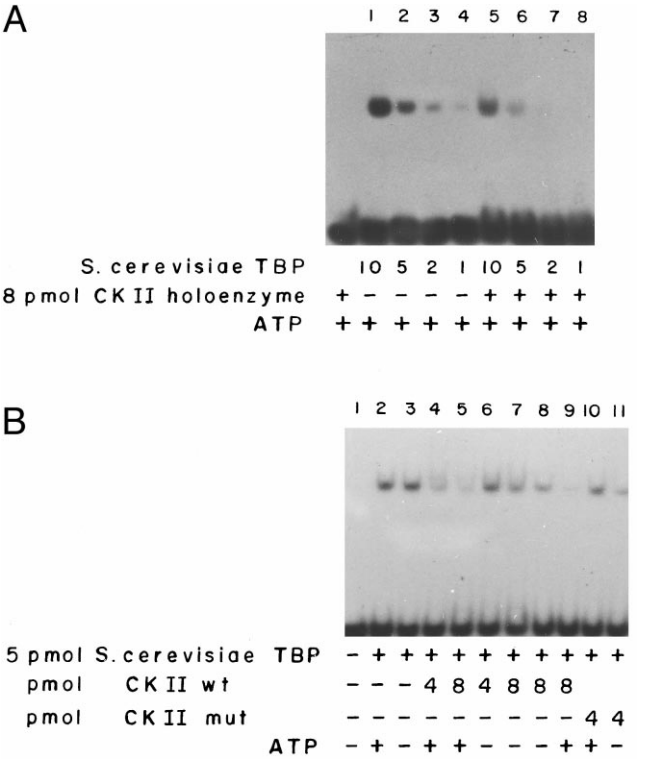

Fig. 2. Inhibition of the binding of TBP to the TATA box by phosphorylation by CK2. A: Different amounts of recombinant *S. cerevisiae* TBP (1, 2, 5 and 10 pmol) were incubated with 0.5 mM ATP in the presence or absence of CK2 holoenzyme. B: Recombinant *S. cerevisiae* was incubated with wild type (wt) or mutant (mut) CK2 holoenzyme in the presence or absence of 0.5 mM ATP. The binding reactions contained 50 ng of recombinant human TFIIA and the Ad-MLP TATA motif and they were performed as described in Section 2. The products of the reactions were analyzed using 0.5×TBE gels.

were incubated with recombinant *Xenopus* CK2 subunits α and β in the presence of [^{32}P]γATP under optimal conditions for CK2 activity. In Fig. 1, it is observed that the TBP obtained from both types of yeast is strongly phosphorylated by CK2. It is observed that the slight phosphorylation of the TBP proteins obtained in the presence of the catalytic CK2α subunit alone (Fig. 1B, lane 4 and data not shown) is greatly enhanced by the addition of the regulatory CK2β subunit which is inactive by itself (Fig. 1A, lanes 3–5 and Fig. 1B, lanes 5–8). The recombinant human TBP tested under similar conditions was not phosphorylated by CK2 (data not shown).

3.2. The effect of CK2 phosphorylation on the specific binding of TBP to DNA sequences

The TBP protein can bind specifically the TATA DNA sequence motifs in vitro. This has been demonstrated using a gel retardation assay and specific labelled TATA containing oligonucleotides in the presence of TBP [9].

The effect of phosphorylation by CK2 on the binding of TBP to the TATA box oligonucleotide was studied using gel retardation assays run in the absence of Mg^{2+} and in the presence of the general transcription factor TFIIA, which enhances the binding of the TBP to the TATA box [9].

Fig. 2A shows that the binding of the labelled oligonucleotide to TBP from *S. cerevisiae* is drastically decreased by

preincubation of the TBP with CK2 in the presence of ATP under standard phosphorylation conditions (compare lanes 1–4 with lanes 5–8). As controls, in Fig. 2B similar incubation with CK2α^{A156}, an inactive mutant of CK2α [13] (lanes 10 and 11), or with CK2 in the absence of ATP (lanes 6–8), shows levels of binding of the DNA oligonucleotide sequence similar to those seen with the untreated TBP.

To rule out the possibility that the effect on the binding was due to phosphorylation of TFIIA, we used binding assays in the absence of TFIIA. In the absence of Mg^{2+} in the electrophoretic gel, the binding of TBP requires the stabilization by TFIIA. However, if Mg^{2+} is present in the gels, the binding of TBP to the TATA box is independent of TFIIA. When the experiments are performed in the absence of TFIIA and in the presence of Mg^{2+}, the same effect of CK2 phosphorylation is observed on the binding of *S. cerevisiae* (Fig. 3A, lanes 4–6) and *S. pombe* TBP (Fig. 3B, lanes 3–5) to the TATA box. The addition of ATP alone (Fig. 3A, lane 8 and Fig. 3B, lane 6) or CK2 without ATP (Fig. 3A, lane 7 and Fig. 3B, lane 7) does not cause any effect on the binding of TBP to the TATA box. This indicates that there is a direct negative effect on the binding of TBP to the TATA box due to phosphorylation by CK2.

3.3. CK2α copurifies with mammalian TFIID

Our attempts to phosphorylate human recombinant TBP by CK2 were negative (data not shown). In human cells TBP is part of several different protein complexes. One of those complexes is TFIID which is required for transcriptional initiation and activation by RNA polymerase II. TFIID is composed of TBP and several TAFs. To investigate if TAFs could have an effect on TBP phosphorylation by CK2, we incubated affinity purified human TFIID (composed exclusively of TBP and TAFs) with CK2. Affinity purified TFIID was incubated with [^{32}P]γATP in the presence and in the absence of CK2. Fig. 4 shows that when TFIID was incubated with [^{32}P]γATP a low level of phosphorylation of several TAFs was obtained (lane 4). Addition of the catalytic subunit of CK2 does not significantly increase the level of autophosphorylation (compare lanes 4 and 5). However, if CK2 holoenzyme is added, a

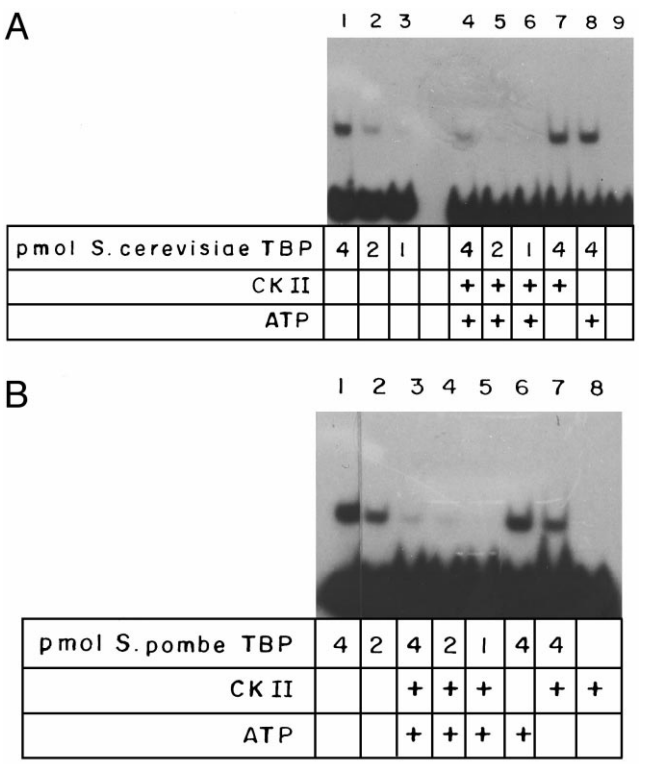

Fig. 3. Inhibition of the binding of TBP to the TATA motif by CK2 is independent of TFIIA. A: Different amounts of recombinant *S. cerevisiae* TBP, as indicated in the figure, were incubated with CK2 holoenzyme and 0.5 mM ATP. B: Recombinant *S. pombe* TBP was incubated under the same conditions as described in A. The phosphorylation reaction was carried out under the conditions used for the binding assay. Binding reactions were done in the absence of TFIIA and contained the Ad-MLP TATA box. The reaction products were analyzed on 5% acrylamide gels which contained 0.5×TBE and 5 mM $MgCl_2$.

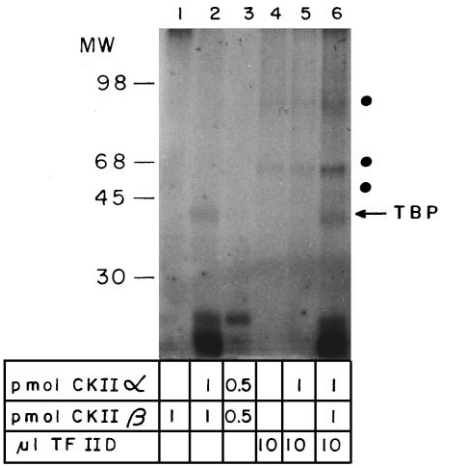

Fig. 4. Phosphorylation of human affinity purified TFIID by CK2. Human TFIID was purified as described in Section 2. An aliquot of 10 μl of the purified complex was incubated under phosphorylation conditions with [^{32}P]γATP in the absence and presence of CK2 holoenzyme. The dots indicate the major polypeptides phosphorylated. The band migrating closely to TBP corresponds to the autophosphorylated CK2α subunit (see lanes 2 and 6). The low molecular weight (~27 kDa) material corresponds to phosphorylated CK2β (see lanes 2, 3 and 6).

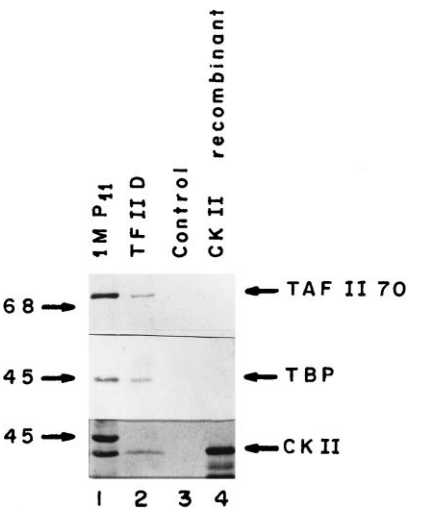

Fig. 5. Western blot analysis of the purified human TFIID complex. An aliquot of the 1 M phosphocellulose derived fraction (5 μl, 2 μg) and 10 μl of the purified TFIID complex were analyzed by Western blot analysis. The blots were incubated with anti-$TAF_{II}70$, anti-TBP and anti-CK2α antibodies, as indicated in Section 2.

higher level of phosphorylation of the same set of TAFs was obtained (compare lanes 4 and 6). The molecular weight of the phosphorylated polypeptides is approximately 55 kDa, 68 kDa and 95 kDa, which may correspond to $TAF_{II}55$, $TAF_{II}70$ and $TAF_{II}100$ respectively. We did not observe phosphorylation of a polypeptide with the size of TBP, and the band which migrates closely to TBP is the autophosphorylated CK2α subunit.

The above results indicate that a kinase with the same specificity as CK2 or CK2 itself may copurify with the TFIID complex. To investigate the latter possibility we used antibodies against the catalytic subunit of CK2 to probe Western blots of affinity purified TFIID. The identity of the TFIID complex was confirmed using antibodies against $TAF_{II}70$ and TBP. Fig. 5 shows that a polypeptide with the same size as CK2α reacts with the antibodies in the TFIID complex (lane 2). This copurification is specific since a control column (which does retain TFIID) does not bind CK2α (Fig. 5, lane 3).

4. Discussion

The results presented above clearly demonstrate that TBP from both budding and fission yeast can be efficiently phosphorylated in vitro by recombinant purified CK2. It is also clear that TBP phosphorylation by the catalytic subunit of CK2 is greatly enhanced (at least 10-fold) by the addition of the regulatory β subunit. The stimulation of the catalytic activity of α by β is observed with the great majority of protein substrates, but with a few such as calmodulin and MDM-2 [14] the addition of CK2β causes inhibition of their phosphorylation.

Our results confirm previous observations with *S. cerevisiae* TBP [6,15] and extend the results to *S. pombe* TBP. Repeated attempts to phosphorylate human TBP with CK2 in vitro failed in our hands. This failure was somewhat surprising due to the fact that the sequence of this protein is highly conserved and that the human TBP contains putative consensus sequences for CK2 phosphorylation. However, we observed that several TAFs of the TFIID complex are phosphorylated by CK2. Moreover, CK2α copurifies with the TFIID complex. It may be possible that in lower eukaryotes CK2 may regulate the specific DNA binding activity of TBP by direct phosphorylation of this protein. However, in higher eukaryotes CK2 may regulate the promoter binding activity of TBP through the phosphorylation of the TAFs, since it is known that TAFs regulate the binding of TBP to the TATA box [16].

The most important new finding presented in this report is the observation that phosphorylation of yeast TBP by CK2 causes a decrease in the binding capacity to the TATA box. It is interesting to note that the negative effect of CK2 phosphorylation of TBP on its affinity of binding to the TATA motif is similar to that noted for several other transcription factors that are phosphorylated by the same kinase. In this regard, transcriptional activators such as c-Max [17], c-Jun [18], Sp1 [19], c-Myb [20] and several others are negatively modulated by CK2. Also, cofactors such as NC2/Dr1 [21] and PC4 [22] are phosphorylated by CK2 and this event negatively regulates their function. However, phosphorylation by CK2 also has a positive effect on the DNA binding activity of several transcriptional activators. The erythroid Krüppel-like factor [23] and MEF2C [24] are phosphorylated by CK2, an event which enhances their DNA binding activity. Taken together, all these findings suggest that CK2 plays a role in the regulation of gene expression. This could be done either directly by the phosphorylation and modulation of the activity of transcription factors or indirectly through the phosphorylation of other kinases involved in the modulation of transcription factors and proteins involved in cellular growth and differentiation. Moreover, it has recently been shown that the binding of the viral transcriptional activator HPV-16E7 to TBP is stimulated upon phosphorylation of E7 by CK2. This suggests that the E7-TBP association and its modulation by CK2 phosphorylation may play a role in cell transformation [25].

It is relevant to consider that CK2 phosphorylation of TBP of *S. cerevisiae* has been found to activate RNA polymerase III transcription by the laboratory of Schultz [15]. The decrease in TATA motif binding described in this report might mean a lowering of RNA polymerase II activity with the concomitant increase in RNA polymerase III and possibly polymerase I activities, in which TBP is not involved in DNA recognition. CK2 activity could, in this hypothetical case, play a role in switching cellular transcription from mRNA synthesis to rRNA and tRNA synthesis. An example of such a switch occurs during amphibian oogenesis as oocytes grow from early stages into the vitellogenic stages. It is significant that *Xenopus laevis* oocytes have been found to contain large amounts of CK2 mRNA and enzyme protein [26].

Currently work is in progress to study further the effects of CK2 phosphorylation on the transcriptional machinery and the factors that control this essential process.

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