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CK2 kinase activity but not its binding to *CK2* promoter regions is implicated in the regulation of *CK2α* and *CK2β* gene expressions

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Abstract Protein kinase CK2, a ubiquitous serine/threonine kinase in control of a variety of crucial cellular functions, is composed of catalytic α- and α′-subunits and non-catalytic β-subunits which form holoenzymes such as $CK2(\alpha\beta)_2$, $CK2\alpha\alpha'\beta_2$, or $CK2(\alpha'\beta)_2$. In addition, there is ample evidence for the occurrence of the individual subunits beside the holoenzyme. While the CK2 subunits are well analyzed on the protein level, only little is known about the regulation of their transcription. The existence of multiple forms of CK2 subunits raised the question about a mutual regulation of their expression. Here we defined two 5′-upstream regions of the *CK2α* and the *CK2β* genes, respectively, as sequences with promoter activities. We found that CK2α and CK2α′ stimulated the expression of the reporter constructs whereas, CK2β was inactive. Using chromatin immunoprecipitation assays, we were unable to detect binding of endogenous CK2 subunits to these promoter sequences in vivo. However, it turned out that inhibition of the kinase activity of CK2 attenuated the promoter activity indicating that CK2α and CK2α′ might regulate their gene expression indirectly by phosphorylation reactions. Thus, we have shown here (i) that under normal physiological conditions CK2 does not bind to *CK2* promoter regions and (ii) that the CK2 kinase activity is implicated in the regulation of its own expression.

Keywords Protein kinase CK2 · Gene expression · Transcriptional regulation · DNA binding

Introduction

Protein kinase CK2, formerly known as casein kinase 2, is known for more than 50 years [1]. It is a ubiquitously expressed protein kinase which phosphorylates more than 500 different substrates and this number is steadily increasing. This large number of different substrates indicates that CK2 is implicated in numerous cellular processes including regulation of cell proliferation and survival [2, 3]. An elevated expression level and an increased kinase activity for CK2 were found in tumor cells compared to normal, non-transformed cells [4]. This later observation made CK2 an interesting pharmacological target for the treatment of cancer [5, 6].

Thornburg and Lindell [7] first described CK2 as a multi-subunit protein kinase that is generated by the association of two α- and/or α′-subunits with a dimer of the β-subunit. This structure and composition of CK2 was quickly confirmed by Dahmus and Natzle [8]. Stigare, however reported in 1993, that CK2α was tightly bound to nuclear structures in the absence of its β-subunit [9] indicating that the CK2 subunits exist not only in the holoenzyme but also in a free form or individually associated with other cellular proteins or structures. An unbalanced expression of CK2α and CK2β subunits was later on described for different mammalian tissues [10–12]. CK2α is not only active as a phosphotransferase in the holoenzyme but also in the absence of CK2β. Loss of CK2β is

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lethal [13] which may imply that some aspects of the CK2β functions are not based on its interaction with the CK2α subunits. Live cell fluorescence imaging revealed that CK2α and CK2β show an independent movement within a eukaryotic cell. Furthermore, CK2α and CK2β enter the nucleus independently [14]. Unlike CK2β, nuclear CK2α can be exported back to the cytosol. Finally, a large excess of CK2β is synthesized compared to CK2α and the non-complexed CK2β protein is rapidly degraded [15]. The human genome contains two *CK2α* loci, at chromosomes 20p13 and 11p15, where primarily the locus on chromosome 20 is transcriptionally active. The locus on chromosome 11 was thought to be a permanently silent pseudogene [16–19]. However, it turned out that this intronless *CK2α* gene is expressed in some cancer cells but only minimally expressed in normal cells [20]. It therefore still has to be clarified whether or not it is a protein-coding gene or a processed pseudogene.

The *CK2α′* gene has been mapped to chromosome 16p13.2–13.3 [21], whereas the *CK2β* gene has been mapped to chromosome 6p21.3 [17]. The genomic structures of the different *CK2* genes in various organisms have been characterized [22–28]. Due to the fact that the individual CK2 subunits assemble into the holoenzyme, which is composed of two CK2α or CK2α′ and two CK2β subunits and that the subunits exist outside of the holoenzyme, it was an obvious question whether the expression of the subunits might be coordinately regulated. The promoter regions for the different *CK2* genes were characterized and furthermore it was shown, that CK2α appears to function as a trans-activating factor for the *CK2β* gene transcription [29]. Electrophoretic Mobility Shift Assays (EMSA) suggested a binding of CK2α to the *CK2β* promoter. Overexpression of CK2α resulted in an elevated level of CK2β protein which might suggest that CK2α stimulate the transcription of *CK2β* although other mechanisms are possible. Previously, it has been shown that CK2 activity is down-regulated at the transcriptional level in both senescent lung fibroblasts and in aged rat tissues and it has been proposed that DNA methylation may be involved in inactivating CK2 in senescent cells [30]. Possibly, promoter methylation may be used as a general mechanism for regulation of CK2 expression. However, so far there is no indication for direct methylation of the *CK2α* and *CK2β* promoters [31].

While the cellular stoichiometry of the catalytic and the regulatory subunits are well characterized, the situation at the transcriptional level is less clear. Thus, we decided to analyze the influence of CK2α, CK2α′, and CK2β on the *CK2α* and *CK2β* promoters. In contrast to previous studies, we used DNA sequences of at least 1.3 kb directly upstream of the translational start sites instead of small DNA fragments. We found that the CK2 catalytic subunits stimulated the expression of both, the *CK2α* and *CK2β* genes. However, using chromatin immunoprecipitation assays, we found no direct binding of CK2α and CK2β to these promoter DNAs. Instead, transcription was attenuated after inhibition of the CK2 kinase activity by two different CK2 inhibitors indicating that the enzyme activity is necessary for transcriptional regulation of the CK2 subunits.

Materials and methods

Cell culture and drug treatment

HCT116 cells are human colon carcinoma cells, which were previously characterized in detail [32] and were a kind gift from Vogelstein [33]. Cells were cultured in McCoy′s 5A Modified Medium with GlutaMAX^{TM}-I (Life Technologies, Darmstadt, Germany) containing 10 % fetal calf serum (FCS; PAA, Pasching, Austria) at 37 °C and 5 % CO_2 in a humidified atmosphere. For selective inhibition of endogenous CK2 kinase activity, HCT116 cells were treated with CK2 inhibitors CX-4945 (1 or 10 μM in DMSO, Selleckchem, München, Germany) or TF (50 μM in DMSO, a kind gift from Joachim José [34]) for 24 h or with DMSO as solvent control.

CK2 protein kinase assay

To study CK2 kinase activity in cell extracts, 30 μg of total protein was mixed with 20 μl of kinase buffer [50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)]. After addition of 30 μl of CK2 assay mix (25 mM Tris–HCl, pH 8.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, 0.19 mM CK2-specific substrate peptide with the sequence RRRDDDSDDD, 10 μCi (0.37 MBq) [γ-^{32}P]ATP), the reaction mix was incubated at 37 °C for 5 min and then stopped on ice. The sample was pipetted onto Whatman-P81 cation-exchange paper and washed three times with 85 mM phosphoric acid for 5 min, followed by washing with ethanol. The dried filter paper was counted for Čerenkov radiation in a scintillation counter (Liquid Scintillation Analyzer 190S AB/LA; Canberra-Packard GmbH, Dreieich, Germany).

Plasmids and cloning of promoter constructs

Firefly luciferase reporter vector (pGL4.10) and *Renilla* luciferase reporter control vector (pGL4.70) were from Promega (Mannheim, Germany). The human *CK2α* (*PI*) promoter fragment, containing 1329 nucleotides upstream of the start codon, was obtained by PCR amplification with the primers CK2α-hum-prom-ATG-1329-for: 5′-ATG AAG CTT GTG CGG TGG CTC ACA TCT GTG-3′;

CK2α-hum-prom-ATG-rev: 5′-ATG CCA TGG GCA TGT CAG ACA GGT TGG CGG AC-3′ and cloned (*Hin*dIII/*Nco*I) into pGL4.10. The human *CK2α* (*PII*) promoter fragment, containing 1920 nucleotides upstream of the first exon, was obtained by PCR amplification with the primers CK2α-hum-prom-up-for: 5′-ATG AGA TCT AGG TAA GCT CCA CCA GTG AGC-3′; CK2α-hum-prom-up-rev: 5′-ATG AAG CTT GCT CTC CCC TCT GCT CAC AC-3′ and cloned (*Bgl*II/*Hin*dIII) into pGL4.10. The human *CK2β* promoter fragment, containing 1896 nucleotides upstream of the start codon, was obtained by PCR amplification with the primers CK2β-hum-prom-for: 5′-AGA AGA TCT CGG GGA GAG TCT C-3′; CK2β-hum-prom-rev: 5′-ATG CCA TGG AGC TGC TCA TCT TCA CGT CAG-3′ and cloned (*Bgl*II/*Nco*I) into pGL4.10. All sequences of cloned fragments were confirmed by DNA sequencing.

The p3xFLAG-CMV-7.1-basic vector was from Sigma-Aldrich (München, Germany). To generate the expression plasmids p3xFLAG-CMV-7.1-CK2α and p3xFLAG-CMV-7.1-CK2α′, the human cDNA of CK2α (1176 nucleotides) and CK2α′ (1050 nucleotides) were cloned into p3xFLAG-CMV-7.1-basic with *Hin*dIII and *Bam*HI. The sequences of both DNA constructs were verified by sequencing. The expression plasmids of the kinase-dead mutants CK2αmut (K68M) and CK2α′mut (K69M) were a generous gift from Litchfield [35]. Both cDNAs were subcloned in p3xFLAG-CMV-7.1-basic vector as described earlier [36]. The *probasin* promoter construct was a kind gift from Thomas Kietzmann and Elitsa Dimova.

Transient transfection, cell lysis, and luciferase assay

Transfection of cells was performed by using the Turbofect® Transfection Reagent (Thermo Scientific, St. Leon-Rot, Germany) according to the manufacturer's instructions.

For the luciferase reporter assay, HCT116 cells were seeded into a 24-well plate (40,000 cells per well) in a total volume of 0.5 ml/well of cell culture medium and cultured overnight. Cells were then transfected using a total of 1 µg of plasmid DNA by using Turbofect® transfection reagent (transfection mixture per well: 0.3 µg of promoter reporter plasmid, 0.2 µg of *Renilla* luciferase control plasmid, 0.5 µg of indicated expression plasmid, 2 µl Turbofect® transfection reagent and 200 µl cell culture medium without FCS).

For western blot analysis, HCT116 cells were seeded into a 6-well plate (800,000 cells per well) in a total volume of 2 ml/well of cell culture medium. Cells were cultured overnight and were then transfected using Turbofect® transfection reagent using a total of 4 µg of plasmid DNA (transfection mixture per well: 1 µg of promoter reporter plasmid, 1 µg of *Renilla* luciferase control plasmid, 2 µg of indicated expression plasmid, 6 µl of Turbofect® transfection reagent and 400 µl of cell culture medium without FCS).

For measuring luciferase activity, cells were collected at given time points after transfection by lysing in passive lysis buffer (PLB, Promega) and measured with the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's recommendations. Relative light units (RLUs) were calculated by normalizing *firefly* luciferase activity to *Renilla* luciferase activity.

For western blot analysis, cell lysates were centrifuged at 13,000×*g* to remove cell debris. The protein content was determined with the Roti®-Quant kit (Roth, Karlsruhe, Germany). Protein extracts were immediately used for western blot analysis or stored at −20 °C.

SDS-polyacrylamide gel electrophoresis and western blot analysis

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis following standard protocols [37]. In brief, 50 µg of proteins per lane were dissolved in sample buffer (130 mM Tris–HCl, pH 6.8, 0.02 % bromophenol blue (w/v), 10 % β-mercaptoethanol, 20 % glycerol (v/v), and 4 % SDS (w/v)) and separated on a 12.5 % SDS-polyacrylamide gel in electrophoresis buffer (25 mM Tris–HCl, pH 8.8, 192 mM glycine, and 3.5 mM SDS) and transferred onto a PVDF membrane (Roche Diagnostics, Mannheim, Germany) in a buffer containing 20 mM Tris–HCl, 150 mM glycine, pH 8.3. The membrane was blocked with 5 % dry milk in PBS with 0.1 % of Tween20 (PBST) for 1 h and then incubated with appropriate primary antibodies diluted in PBST with 1 % dry milk (incubation buffer). For the detection of protein kinase CK2, we used rabbit anti-peptide serum #26 (α-subunit) [38], rabbit polyclonal anti-CK2β serum and the monoclonal antibody anti-FLAGM2 (F3165, Sigma-Aldrich). We used an α-tubulin-specific mouse monoclonal antibody (clone DM1A, Sigma-Aldrich) as loading control. All the antibodies were diluted 1:1,000 in incubation buffer and incubated with the membrane for 1 h at room temperature or overnight at 4 °C. The membrane was washed twice with incubation buffer and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG, 1:30,000 or goat anti-mouse IgG 1:30,000 in incubation buffer; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Chemiluminescence signals were visualized by the ECL Lumilight system of Roche Diagnostics according to the manufacturer's instructions.

Chromatin immunoprecipitations (ChIP)

ChIP assays were performed with minor modifications as described earlier [39]. 1 % of crosslinked and sonicated chromatin of HCT116 cells was taken as input and kept at 4 °C overnight instead of precipitation. DNA from approximately 2.5 × 10^6 cells was used in each immunoprecipitation

with the following antibodies: anti-CK2α, rabbit anti-peptide serum #26 [38]; anti-CK2β, rabbit polyclonal antibody against full length protein; anti-SP1 rabbit polyclonal antibody (07–645, Merck Millipore, Darmstadt, Germany); normal rabbit IgG (sc-2027, Santa Cruz Biotechnology), used as a control for non-specific signals. For the precipitations, 1 µg of normal rabbit IgG, 4 µg of antibody anti-SP1, and 20 µl of anti-peptide sera were utilized. After addition of the antibodies, the samples were incubated at 4 °C overnight on a rotating wheel. Twenty-five microlitre of Protein G Dynabead suspension (Life Technologies) were used to purify antibody-bound protein–DNA complexes. The beads were washed seven times with wash buffer (0.25 M LiCl, 0.5 % (v/v) Nonidet P-40, 0.5 % (w/v) sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0) before the bound complexes were eluted twice, each with 100 µl elution buffer (50 mM Tris–HCl, pH 8.0, 0.1 % (w/v) SDS, 10 mM EDTA) at 28 °C. After elution, formaldehyde crosslinks were reversed and DNA was precipitated and purified. qPCR was then carried out using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix kit (Agilent Technologies, Böblingen, Germany) with the following primers (Fig. 1a, b): ChIP-CK2α hum for: 5′-GGG AGG TTC GTG TTC TCT CT-3′, ChIP-CK2α hum rev: 5′-GGA ATC TCT CTG AAT GCG GC-3′; ChIP-CK2β hum for: 5′-CCA TAA GGA CCC AGC GGA TTG-3′, ChIP-CK2β hum rev: 5′-CAA CGA GAA GCC ACC GGA AG-3′, ChIP-GAPDHS hum for: 5′-AGA CCA GCC TGA GCA AAA GA-3′, ChIP-GAPDHS hum rev: 5′-CTA GGC TGG AGT GCA GTG GT-3′. Product amplification was carried out with an annealing temperature of 62 °C for 40 cycles using 1 µl of DNA solution as template in the PCR Reader Mx3000Pro™ (Agilent Technologies). All PCR results were normalized to input controls.

Statistical analysis

Results of luciferase reporter assays in Fig. 2a, d were expressed as mean ± standard deviation (SD) of at least eight independent experiments. Statistical analysis of the data was performed using the Two-sample *t*-test (Origin software; OriginLab Corporation, Northampton, MA, USA). $p < 0.05$ was considered significant.

Results

The relevant promoter regions of CK2 subunit genes reside upstream of their respective transcriptional start sites

For the analysis of transcriptional regulation of CK2 subunit expression, relevant promoter regions have to be identified. Especially the organization of the *CK2α* gene with the first untranslated exon located over 35 kb upstream of its translational start site emerges the question, which DNA region harbors the relevant promoter region of this gene. Therefore, in contrast to previous studies [29], we amplified extended DNA regions of over 1.3 kb directly upstream of the *CK2α* start codon (*PI*, Fig. 1a) as well as nearly 2 kb immediately upstream of the distant transcriptional start sites of the *CK2α* gene (*PII*, Fig. 1a) by PCR and cloned both potential *CK2α* promoter fragments into luciferase reporter vector pGL4.10. For analysis of the transcriptional regulation of the *CK2β* gene, we amplified and cloned a DNA fragment encompassing 1.9 kb upstream of the *CK2β* start codon, including the transcriptional start site, the first untranslated exon 1 as well as intron 2 of the *CK2β* gene (Fig. 1b).

HCT116 cells were transfected with the reporter constructs together with a *Renilla* luciferase plasmid to calculate the transfection efficiency. As a further control, we also transfected the vector DNA without the putative promoter regions into HCT116 cells. Cells were extracted and the luciferase activity was measured as described in "Materials and methods" section. The luciferase reporter gene assays reveal clearly that only those DNA sequences directly upstream of the transcriptional start site of *CK2α* and *CK2β* genes, *PII* and *CK2β*, exhibit very strong promoter activities (Fig. 1c). The sequence immediately upstream of the translational start site (*PI*) showed no promoter activity. Therefore, no further experiments were carried out with this inactive reporter construct. For simplicity, the active promoter construct *CK2α* (*PII*) is hereinafter called *CK2α* promoter. In the next step, we analyzed whether the reporter assay is dose dependent. Therefore, 50, 100, or 300 ng of the promoter constructs were transfected into HCT116 cells. The following reporter assay revealed that the luciferase activity increased with increasing concentrations of the reporter constructs (Fig. 1d). The relative luciferase light units were displayed relative to the vector control.

Ectopic expression of catalytic CK2 subunits but not of their kinase-dead mutants leads to a modest but significant increase in promoter activity of *CK2α* and *CK2β* promoters

Regulation of *CK2α* and *CK2β* gene expressions at the transcriptional level is not well characterized to date. More than 20 years ago CK2α was found to bind to calf thymus DNA and to λ-phage DNA [40]. Later on, the CK2α protein was found to complex with the *CK2β* gene promoter [29]. Therefore, we decided to analyze whether the catalytic subunits of CK2 (CK2α or CK2α′) might influence the expression of CK2 subunits. To this end, we transiently transfected cells with an active reporter construct

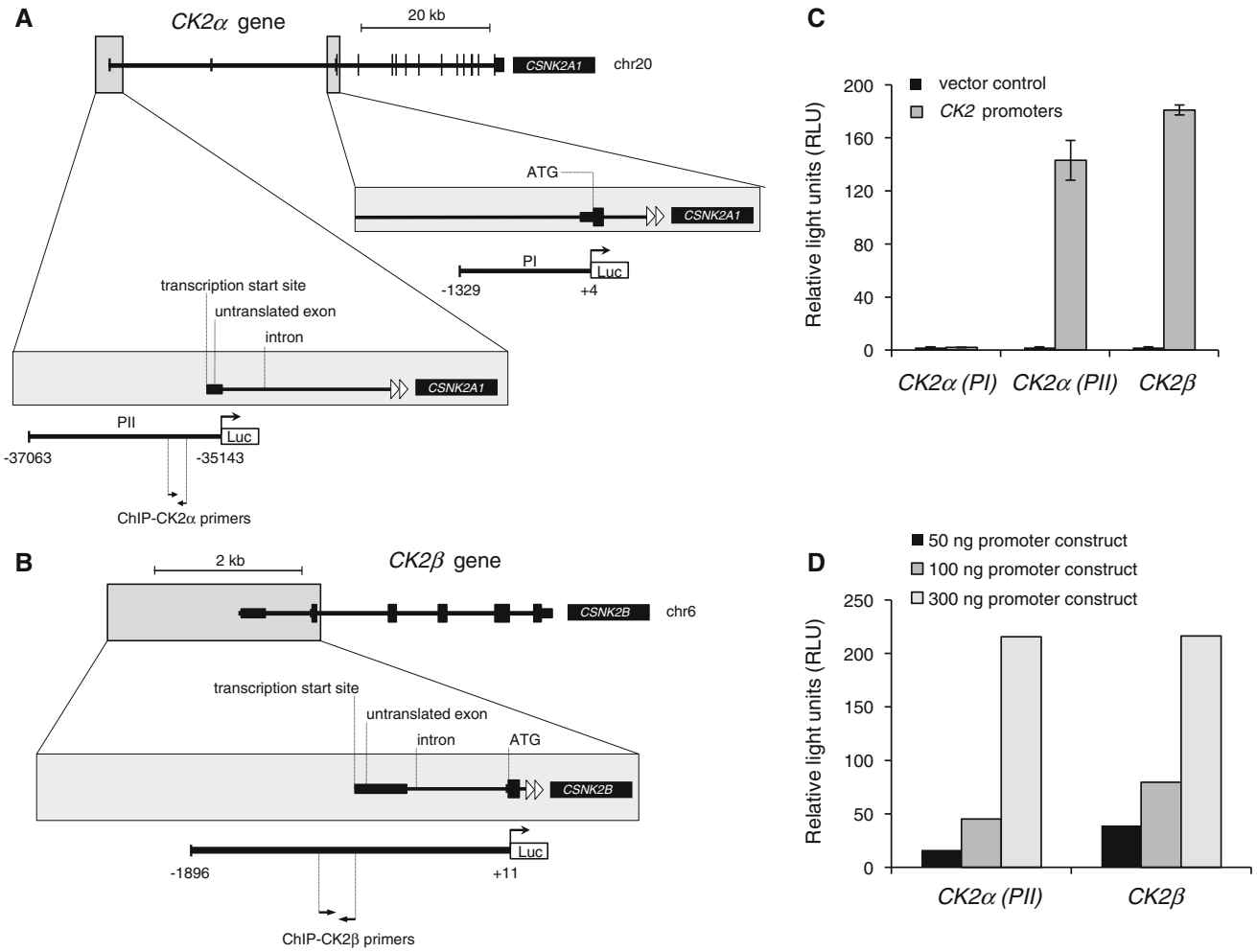

Fig. 1 Genomic architecture of *CK2* genes and identification of relevant promoter regions. Positions of transcription start sites, exons, introns, start ATGs, and primers used for ChIP analyses (Fig. 4) are shown. **a** Potential promoter regions (*PI* and *PII*) of *CK2α* gene are enlarged and cloned DNA-fragments are depicted by *black bars* in front of the coding sequence of the *firefly* luciferase gene (Luc). Nucleotide positions relative to the original translational start site of *CK2α* gene are indicated. **b** The potential promoter region of the *CK2β* gene is enlarged and the cloned DNA-fragment is depicted by a *black bar* in front of the coding sequence of the *firefly* luciferase gene (Luc). Nucleotide positions relative to the original translational start site of the *CK2β* gene are indicated. **c**, **d** Dual luciferase reporter gene assays were carried out in HCT116 cells after transient transfection of 250 ng or indicated amount of different *CK2* promoter constructs. **c** Empty firefly luciferase reporter vector (pGL4.10) served as vector control. Relative luciferase light units (RLU) were displayed relative to the vector control. Mean ± SD, $n = 3$. **d** Relative luciferase light units (RLU) of one representative experiment are shown

(*CK2α-luc* and *CK2β-luc*, respectively), together with plasmids coding for CK2α, CK2α′ or an empty vector control. Our data clearly demonstrated a modest but significant increase in *CK2α* as well as *CK2β* promoter activities after ectopic expression of CK2α (Fig. 2a) and CK2α′ subunits (Fig. 2d). These trans-activating effects observed 24 h after transfection increased further when incubation time was extended to 48 h. No further increase was observed 72 h after transfection (Fig. 2a, d). Western blot analysis with aliquots of transfected cells demonstrated protein expression of FLAG-tagged CK2α and CK2α′ subunits, respectively (Fig. 2a, d) demonstrating that similar amounts of CK2α or CK2α′ were used for the determination of the luciferase activity. In contrast, ectopic expression of kinase-inactive mutants of CK2α(K68M) or CK2α′(K69M) [35] significantly influenced neither *CK2α* nor *CK2β* promoter activities (Fig. 2c, f), although cotransfection of expression plasmids for these mutants lead to a clear increase in the respective protein level (Fig. 2c, f). As a further control, we analyzed effects of ectopic expression of FLAG-tagged CK2α and CK2α′ subunits towards (i) the *probasin* promoter and (ii) the empty reporter plasmid pGL4.10. We found that expression of CK2α or CK2α′ had no influence, neither on the empty reporter plasmid (pGL4.10) nor on the *probasin* promoter (Fig. 2b, e).

As CK2α and CK2β form heterotetramers, we additionally analyzed promoter activities after co-expression of both

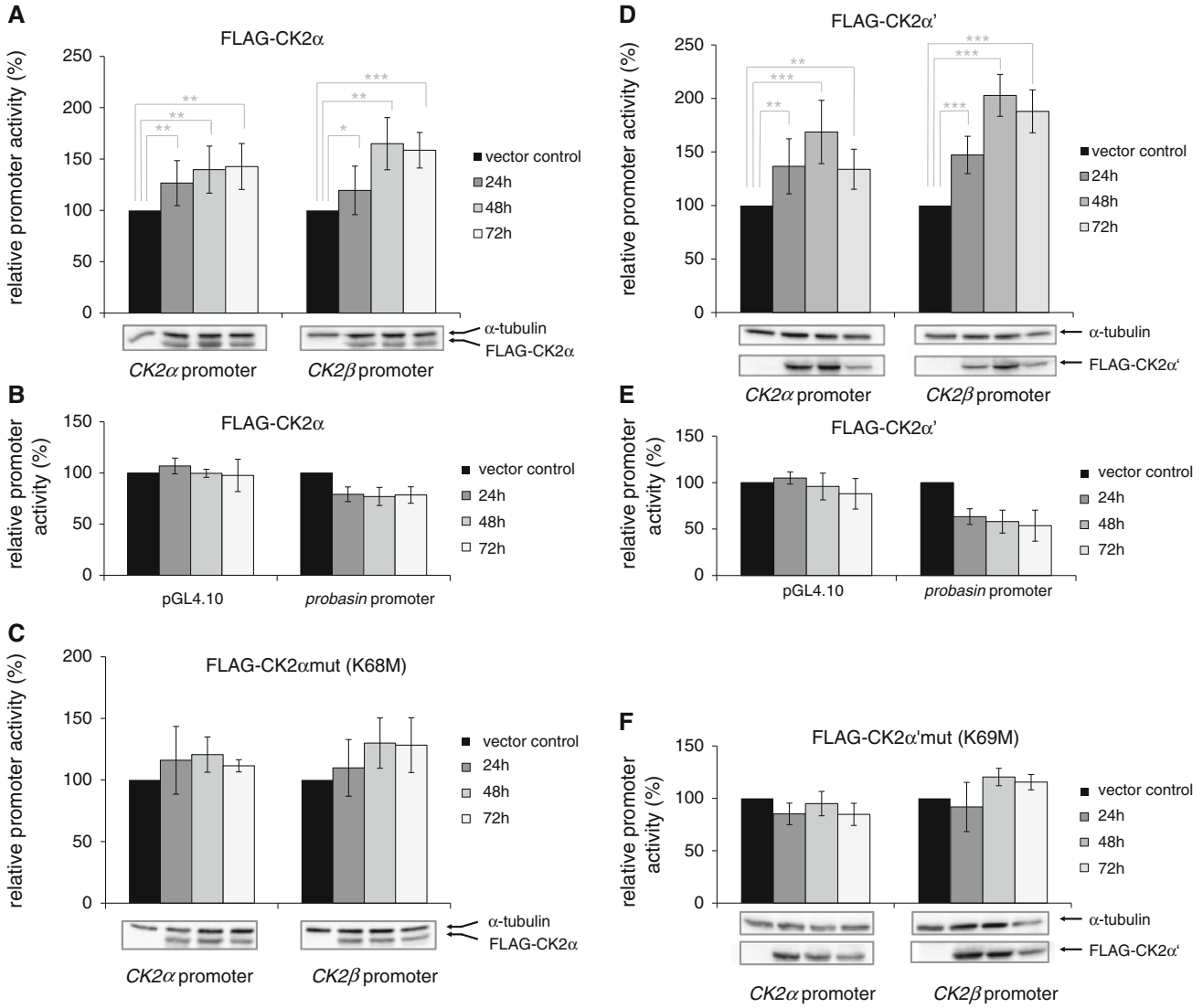

Fig. 2 Ectopic expression of catalytic subunits of CK2 significantly increases promoter activities of *CK2α* and *CK2β* promoters. Dual luciferase reporter gene assays were carried out in HCT116 cells after transient cotransfection of expression plasmids of wild-type CK2α (**a**) or CK2α′ (**d**) or kinase-dead mutants of CK2α (**c**) or CK2α′ (**f**) together with indicated *CK2* promoter constructs or an irrelevant promoter construct (*probasin* promoter construct) or empty reporter plasmid (pGL4.10) as negative controls (**b**, **e**). The empty expression plasmid p3xFLAG-CMV-7.1 served as vector control in each case. Relative promoter activities measured 24, 48, or 72 h after transient transfection, are presented here. *CK2* promoter activities measured after cotransfection with the vector control were set to 100 %. Means and standard deviations of eight independent experiments are shown. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Western blot analyses of cotransfected HCT116 cells were analyzed in parallel and illustrate expression of FLAG-tagged wild-type CK2α (**a**), FLAG-tagged CK2αmut (**c**), FLAG-tagged wild-type CK2α′ (**d**), or FLAG-tagged CK2α′mut (**f**). Anti-FLAG antibody was used at a dilution of 1:1,000. Detection of α-tubulin protein by anti-α-tubulin antibody, at a dilution of 1:1,000, was used as a loading control for the extracts in the experiments

CK2α/CK2α′ and CK2β subunits, alone and in combination. As expected, ectopic expression of CK2β alone did not change CK2 promoter activities significantly (Fig. 3a), whereas CK2α and CK2α′ led to a moderate but significant increase of both promoter activities (Fig. 3b, c). Surprisingly, neither *CK2α* nor *CK2β* promoter activities increased further after combined overexpression of CK2α and CK2β subunits (Fig. 3b). Also combined expression of CK2α′ and CK2β subunits, did not significantly change *CK2α* promoter activity (Fig. 3c). Solely, the activity of the *CK2β* promoter seemed to be slightly increased after combined CK2α′ and CK2β expression. Taken together, these data indicate that obviously monomeric CK2 rather than the holoenzyme is responsible for the observed autoregulation of CK2 expression.

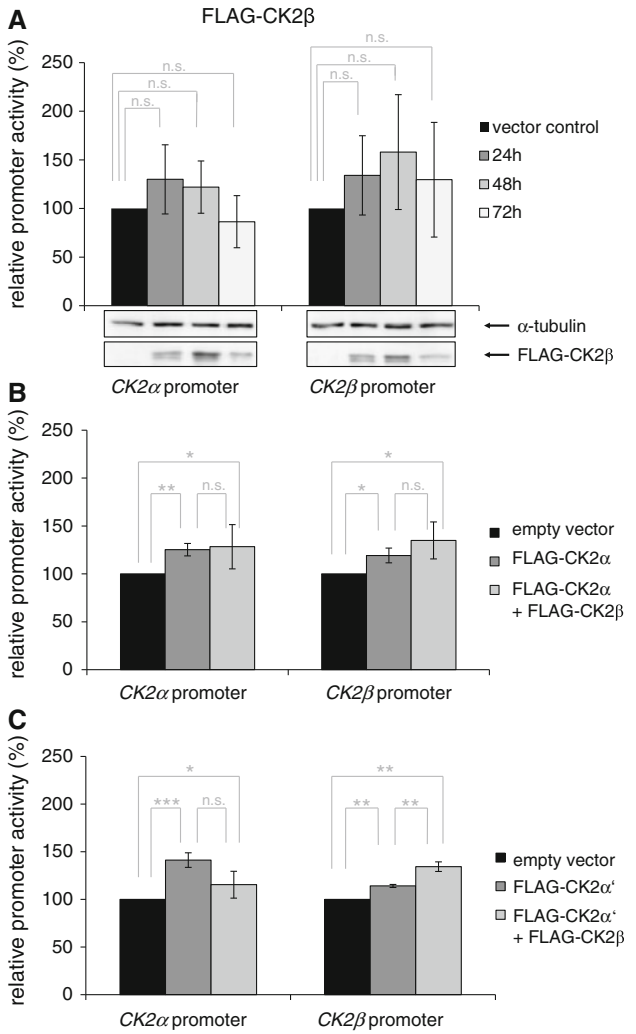

Fig. 3 Ectopic expression of non-catalytic subunit of CK2 did not significantly change promoter activities of *CK2α* and *CK2β* promoters. Dual luciferase reporter gene assays were carried out in HCT116 cells after transient cotransfection of wild-type CK2β alone (**a**), CK2α together with CK2β (**b**), or CK2α′ together with CK2β (**c**) expression plasmids together with indicated *CK2* promoter constructs. The empty expression plasmid p3xFLAG-CMV-7.1 served as vector control in each case. Relative promoter activities measured 24, 48, and 72 h (**a**) or only 24 h (**b**, **c**) after transient transfection, are shown. *CK2* promoter activities measured after cotransfection with the vector control were set to 100 %. Means and standard deviations of three independent experiments are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Western blot analyses of cotransfected HCT116 cells were analyzed in parallel and illustrate clear overexpression of FLAG-tagged wild-type CK2β (**a**). Anti-FLAG antibody was used at a dilution of 1:1,000. Detection of α-tubulin protein by anti-α-tubulin antibody, at a dilution of 1:1,000, was used as a loading control for the extracts in the experiments

Transcriptional regulation of CK2 subunits expression through CK2α and CK2β is not dependent on their direct binding to promoter DNA

As expression of the CK2α subunit leads to an increase in promoter activities of their respective genes, we wondered whether the CK2α subunit might influence *CK2α* and *CK2β* transcription through direct binding to their promoter regions. To analyze direct binding of CK2 subunits to the respective *CK2* promoter regions in vivo, we performed chromatin immunoprecipitation (ChIP) experiments. It was previously shown that the general transcription factor SP1 binds to a minimal fragment of the *CK2α* promoter [41] as well as to the *CK2β* promoter [42]. Therefore, serving as a positive control in our ChIP assay, endogenous SP1 was found to bind to *CK2α* as well as *CK2β* promoter regions in vivo (Fig. 4). In contrast to SP1, neither endogenous CK2α nor CK2β were able to bind *CK2α* or *CK2β* promoter DNA in vivo (Fig. 4). As a negative control, none of these proteins bind to the *testis-specific glyceraldehyde-3-phosphate dehydrogenase* promoter (*GAPDHS*), indicating specific binding in the ChIP assays.

Promoter activities of *CK2α* and *CK2β* decrease after inhibition of CK2 kinase activity

After exclusion of a direct binding of CK2 subunits to their respective promoter regions, we next asked whether the CK2 kinase activity might have an influence on the transcriptional regulation of *CK2α* and/or *CK2β*. To address this question, we transfected HCT116 cells with *CK2α* and *CK2β* promoter constructs, subsequently, we blocked endogenous CK2 kinase activity through treatment of the cells with CX-4945, a highly selective inhibitor of CK2 kinase activity. It was shown earlier that sensitivity of cancer cell lines to CX-4945 can vary in a broad range [43]. Therefore, in early experiments we treated HCT116 cells with CX-4945 at different concentrations. Only about 65 % of endogenous CK2 kinase activity of HCT116 cells was blocked after treatment with 1 μM of CX-4945, while treatment of these cells with 10 μM of CX-4945 lead to a nearly complete inhibition of endogenous CK2 kinase activity (Fig. 5b). We therefore decided to use this CK2 inhibitor at a concentration of 10 μM for inhibition of endogenous CK2 kinase activity. As shown in Fig. 5 about 90 % of endogenous CK2 kinase activity was inhibited after treatment of the HCT116 cells with CX-4945 for 24 h (Fig. 5b). Furthermore, promoter activities of both *CK2α* and *CK2β* promoters were repressed in a dose-dependent manner when endogenous CK2 kinase activity was blocked by the selective CK2 inhibitor CX-4945 (Fig. 5a).

We further treated HCT116 cells with the structurally unrelated CK2 inhibitor TF [34]. At a concentration of 50 μM TF, we found a decrease in endogenous CK2 kinase activity (Fig. 5e) comparable to the effect of treatment of the cells with 1 μM CX-4945 (Fig. 5b). Analyses of *CK2* promoter activities (Fig. 5d) and endogenous *CK2* mRNA levels after treatment of the cells with TF (Fig. 5f),

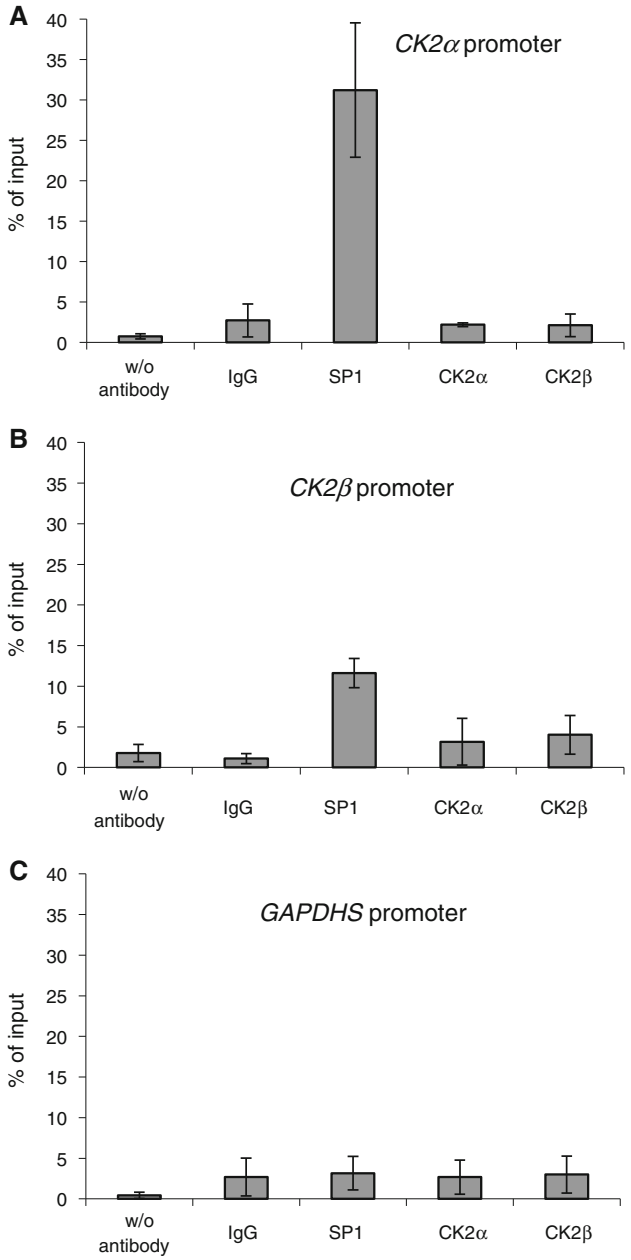

Fig. 4 Neither CK2α nor CK2β subunits are able to bind in vivo to *CK2α* or *CK2β* promoter regions. Nuclear extracts were prepared and chromatin immunoprecipitation analysis of CK2 subunit binding to the human *CK2α* (**a**) or *CK2β* promoter (**b**) in vivo was performed with antibodies targeting CK2α or CK2β. Primer positions are shown in Fig. 1a, b. As negative controls, either no antibody (w/o antibody) or a non-targeting rabbit antibody (IgG) was used. Analysis of SP1-binding served as a positive control. All signals are given relative to the input. **c** The *GAPDHS* promoter served as negative control for SP1 binding

revealed similar effects as they are induced by 1 μM CX-4945 (Fig. 5a, c).

Taken together, our data suggest, that CK2α and CK2α′ influence the expression of *CK2α* and *CK2β*, on the transcriptional level not directly by binding to DNA but by the CK2 kinase activity.

Discussion

The regulation of protein kinase CK2 has been the subject of intensive research [44, 45]. Previous work mainly focused on regulation of CK2 kinase activity and protein level and led to the conviction, that CK2 is a ubiquitously expressed, constitutively active kinase [46, 47]. However, the CK2 kinase has also been shown to be a crucial constituent of tightly regulated cellular processes [48–50]. In addition, the expression and/or the activity of individual CK2 subunits seem to be controlled independently but very precisely. This is evidenced inter alia by differences in the relative expression of individual CK2 transcripts during embryonic development as well as by differential expression of *CK2* genes in diverse tissues of adult animals [48, 51, 52]. For example, CK2α is widely expressed in all human tissues while CK2α′ is predominantly expressed in testis and in brain [10, 53]. Furthermore, the expression of CK2β protein differs from that of CK2α and CK2α′ proteins, suggesting variations in the composition of the tetrameric CK2 holoenzyme in different tissues [54]. The underlying mechanisms for such differences may comprise transcriptional and translational regulation, posttranslational modifications, and stabilization of CK2 subunits at both mRNA and protein level, as well as the subcellular localization of individual CK2 subunits or CK2 holoenzyme.

Little is known to date about the transcriptional regulation of CK2 subunit expression. In an early report, it was shown that CK2 bound to calf thymus DNA and to λ-phage DNA [40]. Blotting experiments showed that DNA bound CK2 through its α-subunits. Pyerin et al. [29, 41, 42, 55] characterized DNA sequences upstream of the transcriptional start sites as promoter regions and used different fragments for promoter studies. Using gel mobility shift-assays as well as DNaseI footprinting, CK2α was found to bind to fragments within these promoter sequences but only when CK2α was expressed in excess of CK2β [29].

To address this issue, we decided to analyze transcriptional regulation of individual CK2 subunits with the focus on potential self-regulatory mechanisms. Previous studies suggested that relevant promoter regions of *CK2α*, *CK2β* genes are located within a few hundred base pairs upstream of the transcription start sites. In most of the experiments, even smaller DNA fragments were used for the analysis of transcriptional control [18, 56]. Interestingly, the *CK2α* gene is organized with the first exon located over 35 kb upstream of the second exon, which harbors the start codon. Several eukaryotic genes are known with a first

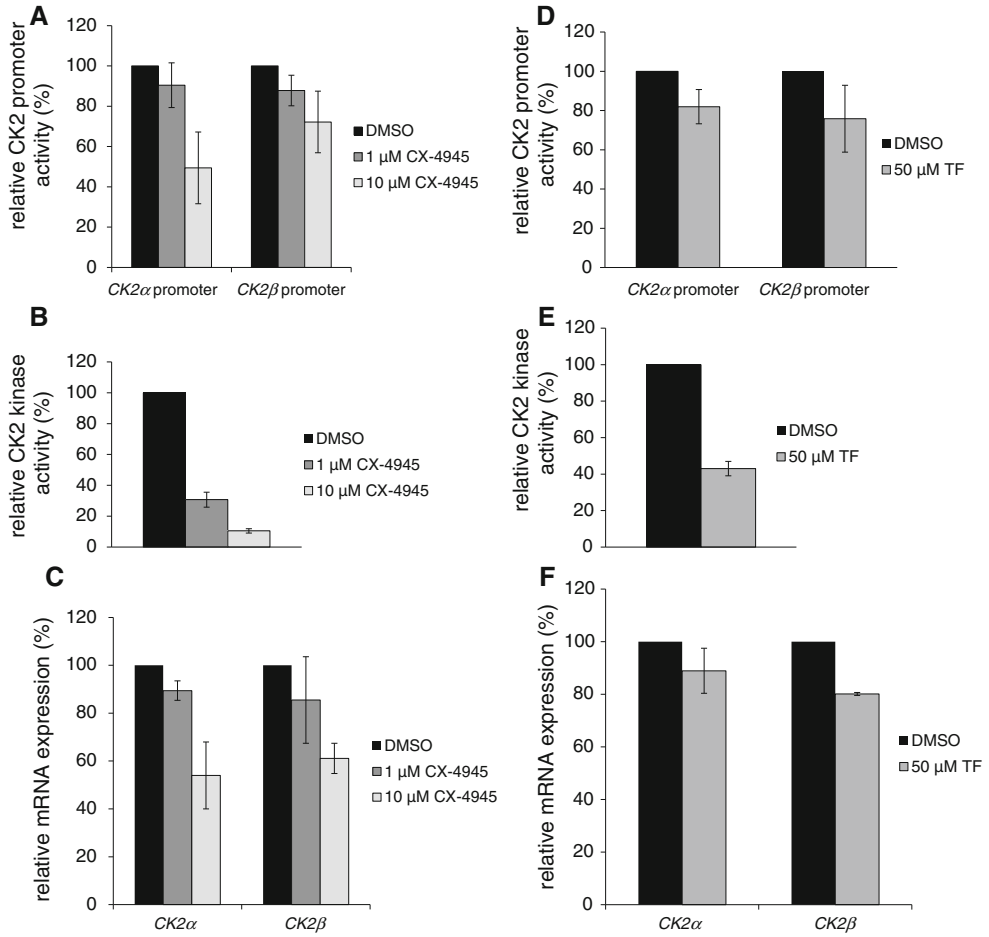

Fig. 5 *CK2* promoter activities and endogenous mRNA levels decrease after inhibition of CK2 kinase activity with selective CK2 inhibitors CX-4945 (**a**–**c**) and TF (**d**–**f**). **a**, **d** Dual luciferase reporter gene assays were carried out in HCT116 cells after transient transfection of indicated *CK2* promoter constructs for 24 h and subsequent treatment with indicated concentrations of the CK2 inhibitors CX-4945 (**a**) or TF (**d**). Relative promoter activities, measured 24 h after treatment of the cells, are presented. *CK2* promoter activities measured in DMSO-treated cells were set to 100 %. **b**, **e** Endogenous CK2 kinase activity in HCT116 cells was determined 24 h after treatment with DMSO (control) or indicated concentrations of CK2 inhibitors CX-4945 (**b**) or TF (**e**). Relative CK2 kinase activities measured in DMSO-treated control cells were set to 100 %. **c**, **f** Endogenous mRNA levels of *CK2α* and *CK2β* were determined after treatment of HCT116 cells with indicated concentrations of CK2 inhibitors CX-4945 (**c**) or TF (**f**). mRNA levels in DMSO-treated control cells were set to 100 %

large intron, which harbor regulatory elements influencing gene expression [57–59]. Furthermore, it is described, that most regulatory elements are located in the 5′-flanking region of the ATG start codon of genes [60, 61]. We therefore were wondered, if there were essential regulatory elements located directly upstream of the ATG start codon of human *CK2α* gene, which may contribute to expression control of CK2α subunit. In order to gain further insights into the transcriptional regulation of the *CK2α* gene, we cloned a potential promoter region proximate to the *CK2α* start codon, encompassing over 1.3 kb (Fig. 1a, *PI*). We however found, that this proximal promoter region does not harbor any promoter activity (Fig. 1c). In contrast, analysis of a distal promoter construct, containing 1.9 kb upstream of the *CK2α* transcriptional start site (Fig. 1a, *PII*), reveals a very strong promoter activity (Fig. 1c). We therefore used this distal *CK2α* promoter construct for subsequent investigations of *CK2α* transcriptional regulation.

The organization of the *CK2β* gene resemble that of *CK2α* concerning location of the start codon within exon 2 (Fig. 1b). The first untranslated exon however, is separated by a smaller intron, encompassing only 612 bp. In order to include all potential regulatory sequences, we cloned a DNA fragment of 1.9 kb upstream of the start codon of the *CK2β* gene. This fragment includes complete sequences of intron 1 and untranslated exon 1 as well as the 5′-flanking region upstream of *CK2β* transcriptional start site. Both, the 1.3 kb upstream sequence of the *CK2α* gene and the 1.9 kb upstream sequence of the *CK2β* gene showed a dose-dependent promoter activity.

We found that the catalytic subunits of CK2 led to a moderate but significant trans-activation of both *CK2α* and

CK2β promoters, whereas the β-subunit did not show any significant transcriptional activation (Figs. 2, 3). According to our data from ChIP analyses, neither CK2α nor CK2β bound to promoter sequences (Fig. 4) which seems to be different from the previous reports. However, Robitzki et al. [29] only observed binding of CK2α to a promoter element when CK2α was present in excess of CK2β. In ChIP assays, we precipitated only endogenous CK2 subunits without any forced overexpression. Under these conditions CK2α is complexed completely to CK2β. Thus, we conclude from our results that CK2α and CK2β do not function as transcription factors for their own expression. This conclusion was supported by the observation that inhibition of the kinase activity by two structurally unrelated inhibitors led to an attenuation of the transcriptional activation of the *CK2α* and *CK2β* genes and to a decrease in CK2 mRNA levels (Fig. 5). Probably due to the well-known high protein stability of CK2 subunits ([15, 62] and our own observation), we were not able to detect this effect on protein level (data not shown). Remarkable, inhibition of over 90 % of endogenous CK2 kinase activity by CX-4945 (Fig. 5b) only leads to downregulation of promoter activities of about 50 % (*CK2α*) or 25 % (*CK2β*), respectively (Fig. 5a). This discrepancy may be explained by basal control mechanisms of *CK2* promoter activities. *CK2α* as well as *CK2β* promoters were basically influenced by several transcription factors, including SP1, ETS1, and NFκB [50, 51, 57]. Obviously, these transcription factors generate basal promoter activities of both promoters, while CK2 kinase activity itself seems to be responsible for the respective fine regulation and required for full promoter activities.

Therefore, our data suggest that CK2 regulates its transcription by phosphorylation of transcription factors. One of the candidates may be SP1 which was shown in this study to bind to both promoter elements and which was shown to be a substrate for CK2 [63, 64]. However, it was shown that phosphorylation of SP1 by CK2 led to a decrease in the DNA-binding activity of SP1 [63]. Thus, we conclude that SP1 is not the transcription factor which is activated by CK2 for the regulation of the CK2 gene expression. Ets1 is another transcription factor which is supposed to bind to the *CK2α* promoter [56]. So far, it is not known whether Ets1 is a substrate for CK2. However, there may be a number of other transcription factors implicated in the regulation of *CK2* gene expression which are not known so far. Work to identify these factors is currently in progress.

We have shown here that under normal physiological conditions CK2 does not bind to *CK2* promoter regions and that the CK2 kinase activity is implicated in the regulation of *CK2α* and *CK2β* gene expressions.

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