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Characterization of Protein Kinase CK2 from *Trypanosoma brucei*

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Abstract

CK2 is a ubiquitous but enigmatic kinase. The difficulty in assigning a role to CK2 centers on the fact that, to date, no biologically relevant modulator of its function has been identified. One common theme revolves around a constellation of known substrates involved in growth control, compatible with its concentration in the nucleus and nucleolus. We had previously described the identification of two catalytic subunits of CK2 in *Trypanosoma brucei* and characterized one of them. Here we report the characterization of the second catalytic subunit, CK2 α' , and the identification and characterization of the regulatory subunit CK2 β . All three subunits are primarily localized to the nucleolus in *T. brucei*. We also show that CK2 β interacts with the nucleolar protein NOG1, adding to the interaction map which previously linked CK2 α to the nucleolar protein NOPP44/46, which in turn associates with the rRNA binding protein p37. CK2 activity has four distinctive features: near equal affinity for GTP and ATP, heparin sensitivity, and stimulation by polyamines and polybasic peptides. Sequence comparison shows that the parasite orthologues have mutations in residues previously mapped as important in specifying affinity for GTP and stimulation by both polyamines and polybasic peptides. Studies of the enzymatic activity of the *T. brucei* CK2s show that both the affinity for GTP and stimulation by polyamines have been lost and only the features of heparin inhibition and stimulation by polybasic peptides are conserved.

Keywords

casein kinase; CK2; protein kinase; Trypanosoma; TAP-tag; nucleolus; NOG1

Abbreviations

5,6-dichloro-1- β -D-ribofuranosylbenzimidazole DRB; IR, infrared; Tet, tetracycline

1. Introduction

The eukaryotic protein kinase CK2, originally called casein kinase II, was one of the earliest identified protein kinases. The kinase primarily consists of two subunits, a catalytic subunit (CK2 α or CK2 α') and a regulatory subunit (CK2 β). The typical stoichiometry is two catalytic

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subunits (homomeric or heteromeric) coupled with two regulatory subunits [1]. Under differing salt conditions, other forms have been observed [2,3]. In addition, there are a number of reports where the subunits function independently of each other, often pairing with other molecules [4–6]. CK2 is distantly related to the CMGC group of protein kinases, particularly the cyclin-dependent kinases (CDKs) [7]. Unlike the CDKs, the activity of the catalytic subunit does not require the presence of the regulatory subunit.

Though extensively studied, the actual role of CK2 has remained elusive. CK2 appears to be essential in every organism tested thus far [8]. Genetic analysis of CK2 function in yeast indicates a role in cell-cycle progression and cell morphology [9,10]. The enzyme is highly pleiotropic, with over 300 known substrates [11]. The largest classes of substrates are involved in either gene expression or protein synthesis, in agreement with the genetic analysis in yeast. The localization of CK2 is consistent with its known substrates. Though dispersed throughout the cell, the protein shows highest abundance in the nucleus, particularly the nucleolus [12–14], which is the site of ribosome biogenesis and the location of many known CK2 substrates.

Confounding the determination of the role of CK2 is its apparently constitutive activation, contrasting with most protein kinases which cycle between an active and inactive state (see [15] and references therein). Regulation of CK2 activity appears to be primarily at the level of protein abundance. Even the regulatory subunit CK2 β does not appear to directly regulate the activity of the enzyme, but rather to modify its interactions with other molecules. While no in vivo effectors of CK2 are known, a number of in vitro effectors have been identified. These effectors include heparin, which inhibits CK2 activity, and polyamines like spermine, which increase activity [16,17]. Polybasic peptides can modulate the ability of CK2 to phosphorylate specific substrates [18–20], which could be relevant in vivo. Another hallmark of CK2 activity is its ability to utilize GTP as well as ATP as a phosphate donor [21]. The physiological significance of this property is not known.

The protozoan parasite *Trypanosoma brucei*, the causative agent for African trypanosomiasis or sleeping sickness, has a complex life cycle involving growth and development in insect and mammalian hosts. The procyclic form (which resides in the insect midgut) and mammalian slender bloodform can be readily cultured in vitro. The genome of the organism has recently been elucidated [22], as has the kinome [23]. Two catalytic subunits of CK2 were identified [23]. One of the subunits, CK2 α (CK2A1, systematic name Tb09.211.4890) was previously characterized [24]. Unlike most other CK2s, the purified *T. brucei* CK2 α subunit, as well as CK2 α immunoprecipitated from the parasites, have only weak affinity for GTP. Here we report the characterization of the second catalytic subunit as well as the identification and characterization of the regulatory subunit CK2 β .

2. Materials and Methods

2.1. Strains and Media

The work described uses either the procyclic *T. brucei* strain 29–13 or the single marker bloodform line, which are derivatives of the 427 strain [25]. Both lines express T7 RNA polymerase and the tetracycline (Tet) repressor allowing for Tet-regulated expression of transfected genes. Procyclic cells were grown in SDM-79 (JRH Biosciences) supplemented with 15% fetal calf serum containing 15 μ g/ml G418 and 50 μ g/ml hygromycin to maintain the T7 RNA polymerase and Tet-repressor genes. Bloodform cells were grown in HMI-9 with 2.5 μ g/ml G418. Both procyclic and bloodform cells were transfected as described [25] with 2.5 μ g/ml phleomycin and/or puromycin 1 μ g/ml added for selection of constructs. Expression from Tet-regulated constructs was induced with 1–2 μ g/ml Tet.

2.2. Plasmids

All primers used for amplification, site-directed mutagenesis and real-time PCR are listed in Table 1. For in vitro expression of proteins, the coding regions of the genes were amplified and cloned into pRSETA. Plasmid pRSETA-CK2 α was described earlier [24]. The CK2 α' and CK2 β coding regions were amplified from genomic DNA with the primers P-CK2 α' 1 and P-CK2 α' 2, and P-CK2 β 1 and P-CK2 β 2 respectively. PCR products for these reactions, as well as all others described, were initially cloned into pGEM-T easy (Promega) and sequenced. Inserts were excised with *Bam*HI and *Xho*I and cloned into pRSETA.

All of the plasmids used for expression in *T. brucei* employed Tet-regulated T7 promoters. To express myc-tagged proteins in *T. brucei*, the CK2A1 gene in pLEW-CK2 α -myc (a pLEW79 derivative) was replaced with ORFs encoding either CK2 α' (CK2A2) or CK2 β (CK2B). CK2A2 was amplified with the primers P-CK2 α' 2 and P-CK2 α' 3. After cloning into pGEM-T easy, the gene was released by digestion with *Hind*III and *Xho*I and ligated into a similarly digested vector. The CK2B gene was excised from the pGEM-T easy plasmid with *Eco*RV and *Xba*I. The target vector was initially digested with *Hind*III and the site filled in with Klenow DNA polymerase. Next, the plasmid was digested with *Xba*I and the CK2 β fragment inserted. For co-expression of CK2 β -myc with other tagged genes, the phleomycin resistance gene was excised with *Spe*I and *Sal*I and replaced with the puromycin resistance gene from pLew79 (PAC)GFP+3'MCS (Saveria, Kessler, and Parsons, submitted).

The plasmid pLEW79-TAP [26] was modified to form pLEW-MHTAP to facilitate cloning and separate the calmodulin binding domain of the TAP tag (which also includes an IgG binding domain from protein A) from the protein of interest. The primers MHTAP S and MHTAP AS were used in site-directed mutagenesis using the Quick-change protocol (Stratagene). The modified plasmid contains a small polylinker (*Avr*II-*Hind*III-*Xho*I-*Bam*HI) in lieu of the luciferase reporter, plus a single myc tag and eight histidine codons upstream of the TAP tag.

To insert the CK2 genes into pLEW-MHTAP, CK2A1 was amplified from genomic DNA with the primers P-CK2 α 1 and P-CK2 α 2, and the fragment was then digested with *Hind*III and *Bam*HI. CK2A2 was amplified with the primers P-CK2 α' 2 and P-CK2 α' 3 and the fragment digested with *Xba*I. Finally, the plasmid pRSETA-CK2 β was digested with *Bgl*II and *Bam*HI. The fragments were then separately ligated into appropriately digested pLEW-MHTAP.

To construct the plasmid pB42AD-CK2 β used for the two-hybrid screen, CK2 β was excised from the pGEM clone used to generate pRSET-CK2 β with *Eco*RI and *Xho*I and ligated into similarly digested pB42AD [27]. The *Saccharomyces cerevisiae* NOG1 gene was amplified with the primers ScNOG1 f1 and r1. The PCR product was re-amplified with primers ScNOG1 f2 and r2. The PCR product was then transformed along with digested pOBD2 as described [28].

For RNAi, gene fragments were inserted into the vector pZJM, which contains head-to-head, Tet-regulated T7 promoters allowing for the expression of double-stranded RNA [29]. Primers P-CK2 α 3 and P-CK2 α 4 were used to amplify a portion of CK2A1. This PCR product, as well as the CK2 α' PCR product described above, were digested with *Xho*I and *Hind*III prior to ligation into *Xho*I-*Hind*III digested pZJM. To simultaneously repress both CK2 α and CK2 α' , the 5' end of CK2A2 was amplified with the primers P-CK2 α' 4 and P-CK2 α' 5 and the product digested with *Xho*I. The fragment was ligated into the *Xho*I digested pZJM-CK2 α plasmid created above to create pZJM-CK2 $\alpha\alpha'$.

2.3. Immunological methods

For generation of antiserum to CK2 α , pRSET-CK2 α was transformed into *E. coli* BL21. CK2 α was found predominantly in inclusion bodies. This fraction was separated by SDS-

PAGE. The region containing CK2 α was cut out and used to immunize rabbits as described [30]. Other primary antibodies and antisera used include E7 anti- β -tubulin monoclonal antibody (mAb) (Developmental Studies Hybridoma Bank, University of Iowa), anti-myc 9E10 mAb (Covance), rabbit anti-HRP (Sigma), and rabbit anti-NOG1 [30].

For immunoprecipitation of proteins generated in vitro, a coupled transcription and translation reaction (TnT, Promega) was used. Templates for protein production included the genes, which were cloned into the *E. coli* expression vector pRSETA (which generates His-tagged proteins), and PCR products containing the untagged ORFs plus a T7 promoter. The latter were generated from the cloned genes using the T7 containing 5' primer CK2 α T7 5' or CK2 β T7 5', matched with the corresponding 3' primers CK2 α 3' TnT or CK2 β TnT 3'. Radiolabeled proteins were generated by adding [35 S]-methionine to the reactions. For immunoprecipitation, Protein A magnetic beads (Dyna) were pre-loaded with anti-CK2 α antiserum at 1:250 and incubated with in vitro translated proteins. Beads were washed three times with SK-lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 2 mM EGTA, 1% NP-40, 0.25% deoxycholate) and samples were analyzed by SDS-PAGE and phosphorimaging.

For immunoprecipitation from *T. brucei*, cells containing inducible expression cassettes were treated with Tet for 16–24 hours and sample lysates prepared as described [31]. Immunoprecipitations were performed as described above, using 10^8 cells per reaction.

Immunoblots followed the protocol described [32] except those imaged by infrared (IR) detection using the Odyssey imaging system (Licor). For infrared imaging, blots were blocked in Odyssey blocking buffer. Blots were subsequently probed with either rabbit anti-CK2 α antiserum (1:5000) or mouse anti- β -tubulin mAb (1:100,000). Primary antibodies were detected with goat anti-rabbit Ig conjugated with AlexaFluor 680 (50 ng/ml) or goat anti-mouse Ig conjugated with IRDye 800 (25 ng/ml) and imaged on the Odyssey.

Cells were fixed and stained for immunofluorescence as described [32]. Antibodies used were 9E10 anti-myc 5 μ g/ml, rabbit anti-HRP (Sigma) 1:200, and rabbit anti-NOG1 1:200 [30]. Primary antibodies were visualized with FITC-conjugated goat anti-mouse Ig or Texas Red conjugated goat anti-rabbit Ig (Southern Biotechnology).

2.4. TAP purification and protein mass spectrometry

Proteins were purified from two liters of cells after overnight induction with Tet. Cells were collected by centrifugation and washed with PBS containing 10 mM glucose. Cells were then lysed in IPP 150 [33,34] containing 0.1% BSA and the protease inhibitors PMSF, leupeptin, aprotinin, E-64, pepstatin, and TLCK at concentrations described [32]. Tagged protein complexes were then purified as described [33]. Briefly, this entails affinity purification on human Ig-sepharose, cleavage of the protein A tag with TEV protease, and subsequent binding and release from calmodulin coupled to agarose column. The resulting proteins have 63 additional amino acids at their C-termini as compared to the native proteins. Preliminary heparin inhibition experiments indicated that additional kinase activities were present in the CK2 α -TAP (but not the CK2 α' -TAP or the CK2 β -TAP) preparations. To remove these activities, the salt concentration was increased from 150 mM NaCl to 1M NaCl in the wash buffer for the Ig-sepharose affinity step (for this protein only). Although NaCl inhibits CK2 α , control experiments showed that this inhibition is reversible (data not shown).

Protein samples were separated by SDS-PAGE on 8–16% gradient gels (Cambrex). Gels were silver stained using SilverQuest (Invitrogen) following manufacturer's protocol and protein bands excised. Excised bands were processed and digested with trypsin as described [34] and analyzed by LC-MS/MS using an LCQ deca XP. Collision spectra were analyzed using Sequest and compared against the *T. brucei* database (www.genedb.org).

2.5. Yeast two-hybrid analysis

Two different yeast two-hybrid systems were used. To analyze the interaction between the *T. brucei* proteins the system described by Estojak et al [27] was used. To analyze the interaction between the *S. cerevisiae* proteins the system described by Uetz et al was used [35].

2.6. Protein kinase assays

Kinase reactions were performed as described [24] using TAP-purified protein, with the addition of casein (5 µg per reaction) or bovine calmodulin (5 µg per reaction) as substrates. Reactions were separated by SDS-PAGE (10% acrylamide) and labeled proteins detected by phosphorimaging. Signals were quantified using ImageQuant (Molecular Dynamics).

2.7. RNA analysis

Northern blots were prepared and hybridized with RNA probes as described [36]. For real-time PCR analysis, RNA was isolated from cells using TRIzol (Invitrogen) and treated with DNase using DNasefree (Ambion) according to manufacturer's protocol. RNA was converted into cDNA using MultiScribe reverse transcriptase (Applied Biosystems). Primers for real-time PCR (900 nM) were CK2α-RT F and R, CK2α'-RT F and R, CK2β-RT F and R and α-tub F and R (α-tubulin). For each real-time reaction, the cDNA generated from 25 ng of RNA was amplified and detected using SYBR Green PCR master mix (Applied Biosystems). Each sample was analyzed in triplicate, normalized against α-tubulin, and referenced against a standard curve generated using genomic DNA.

3. Results

3.1. Identification of CK2α' and CK2β

We previously reported the characterization of a 41 kDa *T. brucei* CK2α catalytic subunit encoded by the *CK2A1* gene and identified *CK2A2* (systematic name Tb927.2.2430), which encodes a distinct, 43 kDa catalytic subunit, CK2α' [24]. As previously noted, both proteins contain the sequence features characteristic of CK2 catalytic subunits, including a conserved tyrosine in region I, a valine in subdomain II, a basic region downstream of subdomain II, and a DWG motif (in lieu of DFG) in subdomain VII. The basic region has been shown to be involved in nuclear localization and substrate recognition of other CK2αs. Reciprocal BLAST analysis indicates that the previously described *Leishmania major* CK2α gene (LmjF35.1730) [37] has the highest homology to *TbCK2A1*, while the *L. major* ORF LmjF02.0360 has the highest homology to *TbCK2A2*. In *Trypanosoma cruzi* a single CK2α gene was identified (Tc00.1047053510761.60), while two CK2α' genes were found (Tc00.1047053508741.320, Tc00.1047053503513.10). The existence of two genes in the *T. cruzi* genome sequence is not surprising since the strain used for the genome project is a hybrid between two distinct genotypes with significant allelic divergence. In the catalytic subunits there is relatively little sequence in addition to the kinase domains but the CK2α' proteins have somewhat longer amino terminal extensions (62 in CK2α' versus 42 amino acids in CK2α in the case of *T. brucei*). Within the catalytic domain, TbCK2α and TbCK2α' respectively have ~ 60% and 50% identity to both human catalytic subunits.

Although most organisms have two types (α and α') of catalytic subunits, usually only one regulatory subunit is found per organism. Querying the genome sequences (www.genedb.org) yielded a single intact CK2β gene in each species (Tb11.01.2590, LmjF36.5090; Tc00.1047053503757.40). Interestingly, *T. cruzi* also possesses a pseudogene, which contains an internal stop codon. The alignment of the three predicted trypanosomatid CK2β proteins with the human, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* proteins is shown in Figure 1. In pairwise comparisons the trypanosomatid sequences showed

over 50% identity amongst each other, and 30–35% sequence identity with human, *S. cerevisiae*, *S. pombe*, *C. elegans*, and *Arabidopsis*. All three trypanosomatid proteins contain the characteristic zinc finger motif found in other CK2 β proteins (overlined in black in Figure 1). CK2 β proteins are typically phosphorylated close to the amino terminus (residues 2 and 3 of the human protein) by the CK2 catalytic subunit [38]. Only the *T. brucei* protein has a consensus CK2 phosphorylation site near its amino terminus (first asterisk, Figure 1).

3.2. CK2 β associates with CK2 α

An antiserum was raised to recombinant CK2 α . This antiserum detected three proteins on immunoblots, the largest and smallest of which were reliably detected. The smallest species comigrated with in vitro translated CK2 α at approximately 38 kDa (Figure 2A). Only the comigrating species was depleted when the level of CK2 α mRNA was reduced by RNAi (Figure 2B). Similarly, it was immunoprecipitated by the antiserum, whereas the other molecules were not (data not shown). Hence the smallest detected band on immunoblots is CK2 α .

To verify that the CK2 β identified was a true homologue, we tested its ability to interact with CK2 α (Figure 2C). After expression in a coupled transcription and translation system, CK2 α and ³⁵S-CK2 β proteins were mixed. As seen in Figure 2C, anti-CK2 α antiserum co-precipitated the labeled CK2 β when CK2 α was present, but not in its absence. We observed in these studies that *T. brucei* CK2 β migrated aberrantly, with the untagged version observed at approximately 44 kDa, as compared to the predicted 34 kDa. The reason for this unusual migration is not clear, although we note the presence of both highly acidic and basic regions in the C-terminal extension unique to this species. In similar experiments using CK2 α' and CK2 β , we were unable to detect any interaction (data not shown). In contrast to CK2 α , in vitro translated CK2 α' was not enzymatically active (data not shown), suggesting that it may be misfolded. We were unable to test the interaction in the yeast two-hybrid system since both CK2 α' and CK2 β were strong transcriptional activators.

We further tested interactions in vivo. When *T. brucei* stable transfectants were induced with Tet to express CK2 β -myc, anti-myc mAb co-precipitated CK2 α , as revealed by immunoblotting (Figure 2D). The experiments were extended by examining TAP-tagged proteins. CK2B, CK2A1 and CK2A2 were cloned into the Tet-regulated TAP tag vector pLEW-MHTAP and procyclic form transfectants containing integrated plasmids were isolated. Following induction, the TAP-tagged proteins, as well as any proteins associated with them, were recovered using the standard TAP procedure of affinity chromatography on immunoglobulin and calmodulin matrices.

Equivalent kinase activities (see below) of the different preparations were separated by SDS-PAGE and silver stained (Figure 3A). Each sample showed a prominent band whose migration corresponded to ~8 kDa larger than the unmodified proteins (8 kDa of the tag remains after proteolytic removal of the protein A domain), although the tagged CK2 α' was relatively less abundant than the other tagged proteins. In the CK2 β -TAP preparation, a second major band was seen, as well as a number of fainter bands. The two major bands and the 44 kDa region were excised, digested with trypsin and processed for protein mass spectrometry. Eight tryptic fragments of CK2 β and a single tryptic fragment of the TAP tag were identified in the 52 kDa band, showing it to contain the purified tagged CK2 β protein. Eleven tryptic fragments of CK2 α were identified in the 40 kDa band. Finally, a single tryptic fragment of CK2 β was identified in the 44 kDa region (the same size as in vitro translated CK2 β), as was a single tryptic fragment of NOPP44/46, a nucleolar protein which has previously been shown to interact with and be phosphorylated by CK2 α [24]. No peptides corresponding to CK2 α' were identified in any of the processed bands.

The CK2 α -TAP and CK2 α' -TAP preparations showed different staining patterns. Neither showed a stoichiometric association with CK2 β . Unlike CK2 β -TAP, where two bands with equal intensity were detected on the silver stain, surprisingly only one prominent band was detected for CK2 α -TAP. To determine if the TAP tag on CK2 α had disrupted the interaction with CK2 β , we co-expressed both CK2 α -TAP and CK2 β -myc in *T. brucei* procyclic forms. TAP pulldowns showed the presence of little CK2 β -myc (Figure 3B), although the protein was clearly expressed as shown by immunoprecipitation with anti-myc mAb (please note, the presence of CK2 α -TAP following immunoprecipitation with anti-myc mAb is uninformative since the TAP tag interacts directly with the Ig reagents). Since Figure 2D shows that CK2 β -myc can interact with endogenous CK2 α , these data suggest that the C-terminal TAP on the catalytic subunits interferes with their interaction with CK2 β . The crystal structure of both the human and maize holoenzymes do not provide any into this finding, since the carboxyl terminus of CK2 α is distal to its site of interaction with CK2 β [39,40].

CK2 α' -TAP expressed very poorly (as did a CK2 α' -myc protein, not shown), so the concentration of the protein kinase eluted in affinity purification was also low. Hence, at least 20-fold more cell equivalents of the CK2 α' preparation were used for gel analysis. Contaminants were correspondingly increased, as seen in the stained gel. The 50 kDa band (asterisk) reacted with anti-myc mAb (myc is contained as an epitope tag upstream of the cleaved TAP tag), showing that it was indeed CK2 α' . In this gel it is difficult to know whether there is any co-purifying CK2 β .

3.3. Characterization of the kinase activity of the purified CK2 enzymes

The CK2 α and CK2 α' preparations enriched by the TAP purification procedure provided a convenient source of CK2 α catalytic subunits largely in the absence of the regulatory subunit. Preparations of CK2 β -TAP protein allowed for characterization of the CK2 $\alpha_2\beta_2$ holoenzyme, although trace amounts of CK2 α' could theoretically be present. We will refer to these preparations as CK2 α , CK2 α' , and holoenzyme, keeping in mind that the composition of the latter is predominantly CK2 $\alpha_2\beta_2$. Initial assays using casein as a substrate indicated that all of the purified proteins were active. The preparations had over a thousand-fold higher protein kinase activity than mock purifications from untransfected procyclic forms (data not shown). In addition to the phosphorylated casein, additional proteins were also phosphorylated and detected in Figure 4A. The identity of most of these proteins is unknown, although the 52 kDa species seen only in the holoenzyme assay likely corresponds to CK2 β -TAP, which was prominent on Coomassie blue-stained gels (see Fig. 3A) and which contains a CK2 phosphorylation site. CK2 α (and the holoenzyme, which contains CK2 α) phosphorylated two electrophoretically distinct subunits of casein, while CK2 α' predominantly phosphorylated only the larger subunit. The basis for this difference was not determined.

One of the key hallmarks of the CK2 kinase is its sensitivity to heparin [16]. We have previously demonstrated that TbCK2 α purified from *E. coli* was inhibited by heparin at nanomolar concentrations [24]. The TAP-purified CK2 α and holoenzyme were also inhibited by nanomolar concentrations of heparin with just 4% and 3% percent activity respectively at 50 nM heparin. CK2 α' was slightly more resistant to heparin inhibition with 31% activity at 50 nM heparin. That over 94% of the activity showed a simple inhibition curve demonstrated that, although not purified to homogeneity, there was only a trace contamination with other kinase activities in the preparations. An additional inhibitor of CK2 activity is 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). We assayed the effect of DRB on isolated CK2 α' and holoenzyme. These had IC₅₀s of 31 μ M and 110 μ M respectively (not shown), in comparison to an in vitro IC₅₀ of 6 μ M for CK2 isolated from HeLa cells [41].

Polyamines generally have a stimulatory effect on CK2 holoenzyme activity in vitro, which is mediated through the CK2 β subunit [17]. We tested the effect of the polyamine spermine on

the *T. brucei* holoenzyme activity. At levels showing the highest activity in other systems, no significant stimulatory effect was seen (Figure 4C).

Polybasic peptides, such as poly-L-lysine, stimulate the ability of CK2 holoenzyme to phosphorylate certain proteins, the prototype of which is calmodulin. We tested whether the *T. brucei* holoenzyme retained this property, using bovine calmodulin as substrate. Poly-L-lysine yielded a 1000-fold stimulation phosphorylation of calmodulin, but had no detectable effect on phosphorylation of the 52 kDa species thought to be CK2 β -TAP (Figure 4D).

One difference between the mammalian holoenzyme and its isolated catalytic subunits is sensitivity to high salt [1]. The activity of CK2 α subunits decrease with increasing salt while the activity of either the $\alpha_2\beta_2$ or $\alpha'\beta_2$ holoenzymes increases three- to four-fold at 50–200 mM NaCl, before dropping at higher concentrations. Like mammalian catalytic subunits, *T. brucei* CK2 α and CK2 α' were both sensitive to salt, losing over 50% of their activity at 100 mM and 200 mM NaCl respectively (Figure 4E). In contrast to the mammalian CK2 holoenzymes, the *T. brucei* holoenzyme showed no salt activation, but rather lost 50% of its activity at 50 mM NaCl (Figure 4E, triangles).

Unlike other protein kinases, CK2 can typically use GTP as well as ATP as a phosphate donor. We have previously reported that *T. brucei* CK2 α has only a very low affinity for GTP [24]. This property is conveyed by the catalytic subunit [42] and, by extension, is exhibited by the corresponding holoenzyme. Excess unlabeled nucleotides were added to the reactions containing CK2 α' to determine if they could compete with the labeled ATP (Figure 4F). Among the four ribonucleotides, only ATP could compete for binding. The deoxyribonucleotide dATP also strongly competed with ATP.

3.4. Nucleolar localization and interactions

We had previously shown that CK2 α concentrates in the nucleolus with diffuse nuclear and cytoplasmic staining. We tested the localization of CK2 α' -TAP and CK2 β -myc. The former was detected by virtue of the binding of the tag to the Fc portion of IgG, while the latter was detected with anti-myc mAb. Unfortunately, the presence of the protein A portion of TAP tag complicates co-localization using antibodies. However, using DAPI as a nuclear stain, a significant proportion of the CK2 α' -TAP can be seen to be nuclear (Figure 5A). It appeared to be concentrated in the region of the nucleus which stains poorly with the DNA dye DAPI, i.e., the nucleolus. However, as expected for a pleiotropic protein kinase, there was additional diffuse cytoplasmic staining (Figure 5A). Similarly, CK2 β -myc showed general cytoplasmic staining with a single region of more concentrated staining (Figure 5B). This region of staining was localized to the nucleolus as seen by the overlay with staining for the nucleolar marker NOG1.

In earlier work, we demonstrated that CK2 α interacts with the nucleolar protein NOPP44/46, which also interacts with the nucleolar GTP binding protein NOG1. The studies presented in Figs. 2 and 3 show that CK2 α and CK2 β interact with one another. We therefore decided to test whether CK2 β interacted with NOG1, using a yeast two-hybrid approach. Indeed the two proteins were found to interact (Figure 6A). The interaction was also detected for the yeast orthologues of these proteins, originally in a genome-wide screen using ScNOG1, and then confirmed by testing the specific ORFs (Figure 6B). We confirmed the interaction in *T. brucei* by co-immunoprecipitation analysis in which the association of CK2 β -myc and NOG1 was tested. CK2 β -myc was expressed in *T. brucei* procyclic forms in the Tet inducible system. We have previously shown that NOG1 is sensitive to proteolysis during extended incubations, such as those required for immunoprecipitations, leading to the generation of multiple immunoreactive species [36]. As shown in Figure 6C, NOG1 was detected in anti-myc immunoprecipitates only when the expression of CK2 β -myc was induced. As expected, it was

detected in all anti-NOG1 immunoprecipitates, whether or not CK2 β -myc was induced. In the reciprocal experiment, anti-NOG1 coprecipitates contained CK2 β -myc.

3.5. Expression of CK2 during the life cycle

T. brucei bloodform parasites proliferate more than two-fold faster than procyclic forms in vitro. When the abundances of the relevant transcripts from both stages were quantified by real-time PCR, only CK2 α mRNA appeared to increase and that increase was modest (average of 67%, Figure 7A). When the abundances of the mRNAs were compared to genomic equivalents, the mRNA abundance of CK2 β was approximately two-fold higher than that of the other two genes. Quantitation of the transcript abundance in slender bloodforms, non-dividing stumpy bloodforms, and procyclic forms from the pleiomorphic strain, TREU667, gave similar results (data not shown). The abundance of CK2 α protein, as revealed by anti-CK2 α antibodies, was comparable between procyclic 29–13 cells and single marker bloodform cells when normalized to β -tubulin, which is constitutively expressed (Figure. 7B). Attempts to raise anti-peptide antisera to CK2 α yielded only low titer antibodies with poor specificity.

3.6. Effect of CK2 depletion on growth

CK2 has been shown to be an essential kinase in most if not all organisms. We investigated its role in *T. brucei* by reducing its expression via RNA interference (RNAi). Regions of either *CK2A1* (nt 1–480) or *CK2A2* (nt 641–1171) were cloned into the RNAi vector pZJM for Tet-regulated expression of double-stranded RNA. Following isolation of stable procyclic form transfectants, RNAi was induced. Surprisingly, the doubling time increased only about 20% (data not shown). We next tested the possibility that there is redundancy between the two kinases by targeting both transcripts simultaneously for RNAi by expressing double-stranded RNA encompassing regions of both genes (nt 1–480 of *CK2A1* plus nt 35–521 of *CKA2*). An additive rather than synergistic effect was seen, with a generation time 40% longer than that of uninduced cells (Figure 8A). The endogenous full-length RNAs at 1.46 kb (CK2 α) and 3.29 kb (CK2 α') were reduced by ~75% at day 2 through day 7 (Figure 8B) following induction. It is clear that the maximal growth rate of *T. brucei* requires CK2 function. Since we saw only a 75% reduction in the mRNA abundances, it remains possible that CK2 activity is essential. Similar findings were seen when double RNAi was performed using bloodstream forms (data not shown). The cell-permeant CK2 inhibitor DRB affected the growth of both stages with 50% inhibition at the highest concentration tested, 200 μ M (data not shown), although off-target effects cannot be excluded. Other than slowed population growth, no phenotype was observed for depletion of CK2 activity by RNAi or DRB treatment.

4. Discussion

The nucleolus, in addition to being the site of ribosome biogenesis, also plays a role in other pathways like gene silencing, cell proliferation, and tumor suppression. Intriguingly, a role for CK2 has been implicated in all of these functions. CK2 is known to accumulate in the nucleus [12] and interacts with several nucleolar proteins [24,43,44]. Here we show that the α' and β subunits of CK2, like the α subunit, are concentrated in the nucleus and nucleolus in *T. brucei*. It has been demonstrated previously that tagged yeast CK2 α 1 is able to pull down the GTP-binding protein NOG1, as well as several other proteins in the pre-ribosome [45], and hence likely associates with the 66S precursor particle. In this report we demonstrate that CK2 β interacts with NOG1 of both *T. brucei* and *S. cerevisiae*, suggesting that the interaction of NOG1 and CK2 β may be one means of associating CK2 with the pre-ribosomal particle. The web of interactions in trypanosomes is extended by our previous work showing that CK2 α interacts with the nucleolar protein NOPP44/46 [24], which in turn interacts with NOG1 [30]. NOPP44/46 also interacts with p34/37 [46], which binds 5S rRNA [47]. It is likely that several of these interactions are transient, perhaps even sequential, rather than all of the proteins

residing together in a stable particle. For example, most of NOG1 resides in the 66S pre-ribosome, whereas NOPP44/46 [36] and CK2 (data not shown) do not. Little is known about CK2 substrates in trypanosomes. In addition to NOPP44/46 [24], tubulin also appears to be a substrate of trypanosomatid CK2 [48].

Despite the use of multiple methodologies, we were unable to determine whether CK2 α' interacts with CK2 β . However, one conclusion that can be made based on the TAP purification is that unlike CK2 α , CK2 α' is not present in high quantities in the CK2 β complexes. This result could be due to a weak interaction or a much lower abundance of CK2 α' protein as compared to CK2 α in procyclic forms (although the abundances of the cognate mRNAs are similar). Our consistent difficulty in obtaining significant expression of tagged CK2 α' even though CK2 α was expressed abundantly using the same promoter, and 5' and 3' UTRs, suggests that expression of this protein may be under tight control.

As mentioned earlier, the distinctive features of CK2 activity include its near equal affinity for both GTP and ATP, modulation by polyamines, activation by salt, sensitivity to polybasic peptides, and inhibition by heparin. The *T. brucei* CK2 isoforms share only the last two traits. Effects of polyamines, salt, and polybasic peptides have been shown to be mediated by a region of CK2 β which has multiple acidic residues (underlined in Figure 1). Photoaffinity labeling studies using a spermine analog demonstrated covalent linkage to a residue within aa 72–78 of *Drosophila* CK2 β [49], which contains two acidic residues conserved in TbCK2 β . When three nearby acidic residues were mutated to alanine, the *Drosophila* enzyme showed higher basal activity and less stimulation by spermine [50]. These three acidic residues (60, 61, 63 in human and *Drosophila*, filled circles on Figure 1) are not conserved in *T. brucei*. In fact, within the “acidic” region, two basic residues are found in the trypanosomatid CK2 β s. Hence the lack of stimulation of CK2 activity by polyamines fits well with data in other systems. The biological implications are unclear since CK2 activity does not appear to be directly modulated by polyamines in vivo [51].

In other organisms, the interaction of CK2 α with CK2 β results in a decrease in enzymatic activity unless salt is added. This finding has been hypothesized to be due to tertiary interactions between the $\alpha_2\beta_2$ tetramers (deduced from secondary contacts seen in the crystal structure), such that two acidic residues of the β subunits contact the active site via ionic interactions [52]. Addition of salt would overcome this interaction. Since the trypanosomatid CK2 β proteins lack these specific acidic residues (see Figure 1, arrows), the holoenzyme would not be expected to show activation by salt. Indeed we saw no evidence for salt activation.

In other systems, the ability of poly-L-lysine to stimulate phosphorylation is specific to certain substrates, of which calmodulin is the prototype. Mutation of acidic residues 55, 57, or the triad 59–61 strongly reduced poly-L-lysine stimulation of human CK2 [53]. Since the *T. brucei* enzyme lacks acidic residues at aa 59–61 (open circles), we did not expect poly-L-lysine to strongly activate CK2 phosphorylation of calmodulin. However, strong activation towards this substrate was observed, comparable to that seen with wild-type human enzyme by the above authors. Calmodulin is a physiological substrate of CK2 [54], and its CK2 phosphorylation sites (T79, S81, S101) are maintained in *T. brucei*. CK2 activation by polybasic peptides is not restricted to poly-L-lysine but can be achieved with proteins such as histones [18–20], suggesting that this conserved property may be physiologically meaningful.

The heparin sensitivity of *T. brucei* CK2 is comparable to that of other CK2s. The final hallmark of CK2, the ability to use GTP as a phosphate donor, is also largely absent in the *T. brucei* enzymes. As reported earlier, one of the catalytic subunits (α) has only a weak affinity for GTP; while here we show evidence that the second subunit (α') has no detectable affinity for GTP. Asn118 in subdomain V has previously been shown to be important in allowing both ATP and

GTP utilization in *Xenopus* CK2 α since its deletion or mutation to Asp reduced the ability to utilize GTP [55,56]. While TbCK2 α ' possesses a Pro at this site (a substitution that was not tested), TbCK2 α has a tolerated substitution (Ala). Recent analysis of human CK2 α has shown that mutation of Val66 to Ala (the residue found in 87% of human protein kinases) and Met163 to Leu (the residue found in 78% of human protein kinases) abrogates the ability to utilize GTP [57]. Val66 is conserved in both trypanosome isoforms, but Met163 is replaced by Ala in CK2 α and Ile in CK2 α '. It seems likely that the inability of the *T. brucei* isoforms to use GTP results from a constellation of differences that combine to alter the purine binding site. Taken together, our results show that *T. brucei* CK2 possesses a number of unusual properties, many of which correlate with structure-function predictions from other systems.

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Figure 1. Alignment of CK2β

Alignment of CK2β from *T. brucei* (Tb11.01.2590), *T. cruzi* (Tc00.1047053503757.40), *L. major* (LmjF36.5090), *H. sapiens* (AAA52123), *C. elegans* (AAA27983), and *S. cerevisiae* (CKB2, P38930). Residues conserved in all shown CK2β sequences are highlighted in black. Dark grey residues are conserved in three of the sequences while light grey are residues similar in at least three sequences. The black line above the sequence is the cysteine Zn⁺⁺ binding motif. The acidic binding region in HsCK2β implicated in both poly-L-lysine and polyamine stimulation is indicated by the grey bar below the sequence. The two arrows denote the location of acidic residues in the human sequence suggested to mediate interactions between tetramers under low salt conditions via binding to basic residues in the active site of CK2α [52]. The filled circles mark residues, which when jointly mutated in *Drosophila* CK2β, result in decreased stimulation by polyamines [50]. Open circles mark three residues which when jointly mutated in human CK2β abrogated the response to poly-L-lysine [53]. Asterisks denote potential CK2 phosphorylation sites in the *T. brucei* protein.

Figure 2. Interaction of CK2α and CK2β

A. Analysis of anti-CK2α antiserum. Full-length untagged CK2α was generated in an in vitro coupled transcription and translation system. The in vitro generated protein (CK2α) was separated by SDS-PAGE, along with a protein lysate from 29-13 procyclic form cells (PCF). After transfer to nitrocellulose, blots were probed with anti-CK2α antiserum. The in vitro translated CK2α comigrates with the 38 kDa band detected by the anti-CK2α antiserum. The two lanes shown were from the same gel, separated by one lane. Arrow points to 38 kDa band comigrating with CK2α.

B. Disappearance of 38 kDa band after depletion of CK2α. Protein lysates generated from cells depleted for CK2α via RNAi (Tet+), see below Figure 7, were run on a gel along with protein lysates from untreated cells (Tet-) and a total cell lysate (TCL) from untransfected cells. Blots were imaged by IR scanning. Arrow points to CK2α. Lower panel shows loading control.

C. In vitro translated CK2α and CK2β interact. CK2α (α) and ³⁵S-methionine-labeled CK2β (β) were generated in an in vitro coupled transcription and translation system. The labeled

CK2 β either mixed with CK2 α or on its own was immunoprecipitated with anti-CK2 α antiserum.

D. Coimmunoprecipitation of CK2 α and CK2 β -myc. Protein lysates made from either induced (Tet+) or uninduced (Tet-) cells were prepared from procyclic cells harboring a Tet-regulated, myc-tagged CK2 β . Either CK2 α (IP-CK2 α) or CK2 β -myc (IP-myc) were immunoprecipitated. Duplicate immunoprecipitates and the total cell lysate (TCL) were separated on an SDS-PAGE gel. Blots of the gel were probed with anti-CK2 α antiserum. The location of CK2 α is indicated by an arrow. The faint 50 kDa band in the CK2 α immunoprecipitate is cross-reacting heavy chain IgG.

Figure 3. CK2 TAP complexes

A. Silver stained gel of samples from TAP purifications. Aliquots of the samples after TAP affinity purification procedure were separated on adjacent lanes of an 8-18% SDS-PAGE and silver stained. The location of the TAP-tagged protein in each lane is marked with an asterisk. Also indicated is the location of the native CK2 α and CK2 β proteins.

B. Coprecipitation of CK2 α -TAP and CK2 β -myc. Protein lysates were made from procyclic cells expressing both CK2 α -TAP and CK2 β -myc. CK2 α -TAP or CK2 β -myc were precipitated and samples separated by SDS-PAGE along with the total cell lysate (TCL). The blot of the gel was probed with anti-myc mAb. The locations of the CK2 α -TAP and CK2 β -myc protein are indicated. Note the absence of CK2 β -myc in the TAP purification. Due to the protein A portion of the TAP tag, CK2 α -TAP is unavoidably bound by secondary antibodies and hence is observed in both the TCL and in the anti-myc immunoprecipitates.

Figure 4. Characterization of the kinase activity of CK2 preparations

A. TAP purified proteins have protein kinase activity. Aliquots of the CK2 α (α), CK2 holoenzyme (holo) and CK2 α' (α') preparations were used in a kinase reaction with casein as the substrate. Reactions were electrophoresed on an SDS-PAGE and stained. After drying the gels were exposed to phosphorimaging screen that was subsequently scanned. For comparison, a portion of the Coomassie blue stained gel is shown (CB). The location of the kinase substrate casein (Cs) is indicated.

B. Heparin inhibition of CK2 activity. The activity of the purified proteins was assessed in the presence of increasing concentrations of heparin. Activities of the various enzymes were normalized to assays done in the absence of heparin.

- C. Spermine does not stimulate CK2 holoenzyme. The stimulatory effect of the polyamine spermine was tested in increasing concentrations with the holoenzyme.
- D. Poly-L-lysine stimulation of CK2 holoenzyme. The activity of the holoenzyme was tested with increasing concentrations of poly-L-lysine with calmodulin used as a substrate. The activity toward both calmodulin (CaM) and CK2 β -TAP (CK2 β) are shown normalized to untreated samples.
- E. Effect of NaCl on CK2 activity. Increasing concentrations of NaCl were added to reactions and normalized against reactions with no added salt.
- F. Nucleotide specificity of CK2 α' . The ability of the CK2 α' catalytic subunit to utilize other nucleotides was tested. Unlabeled nucleotides (1mM) were added to the reactions to determine if they could compete for ATP binding

Figure 5. Localization of CK2α' and CK2β to the nucleolus

A. Localization of CK2α'-TAP. CK2α'-TAP was stained in either induced (Tet+) or uninduced (Tet-) with rabbit anti-HRP, which binds the protein A part of the TAP tag. The stained protein was detected with Texas Red conjugated goat anti-rabbit Ig. The nucleus was stained with DAPI. The images were then overlaid, with red pseudocolor corresponding to TAP and blue to DAPI.

B. Localization of CK2β-myc. CK2β-myc was stained in either induced (Tet+) or uninduced (Tet-) cells with anti-myc mAb and detected with FITC conjugated goat anti-mouse Ig. The nucleolar protein NOG1 was localized with anti-NOG1 antibodies and detected with Texas Red conjugated goat anti-rabbit Ig. The nucleus was stained with DAPI. The NOG1 and CK2β-myc images were overlaid.

Figure 6. CK2β interacts with NOG1

A. Yeast two-hybrid interaction between *T. brucei* proteins CK2β and NOG1. A yeast strain harboring plasmids that allow the expression of both the *T. brucei* NOG1 gene fused to LexA [30] and CK2β gene fused to the Gal4 activation domain was grown in liquid media. The culture was spotted onto media that contained leucine (+Leu) or lacked leucine (-Leu). Growth on the media without leucine indicates that the *LEU2* reporter gene was expressed.

B. Yeast two-hybrid interaction between *S. cerevisiae* proteins CK2β and NOG1. Similar to the above experiment only the *HIS3* reporter gene was used. Shown is the data using the *CKB2* gene fused to the region encoding the Gal4 activation domain constructed by Uetz et al [35]. Similar data was found using the second CK2β gene *CKB1* (data not shown).

C. Coimmunoprecipitation of CK2β and NOG1. Protein lysates were made from *T. brucei* transfectants either induced (Tet+) for expression of CK2β-myc or uninduced (Tet-). Proteins were immunoprecipitated with anti-NOG1 antiserum, the preimmune serum (Pre), anti-myc mAb, or PBS. Immunoprecipitates were separated by SDS-PAGE and proteins transferred to nitrocellulose. The membrane was probed simultaneously with anti-NOG1 and anti-myc reagents. The total cell lysate (TCL) was from induced cells. The faint band denoted by the arrowhead is IgG heavy chain.

Figure 7. Expression of CK2 subunits during the *T. brucei* life cycle

A. Analysis of transcript abundance. RNA from the two proliferative life stages, 29–13 procyclic cells (PCF) and single marker bloodform cells (BF) was isolated and the transcript abundance of CK2 α , CK2 α' , and CK2 β quantified using real-time PCR. Quantities were normalized against α -tubulin transcript abundance. Shown is the data from four independent procyclic and three independent bloodform RNA samples. Each RNA preparation is marked by a different symbol so that the relationships of the subunits in individual samples can be seen.

B. Analysis of CK2 α protein abundance. Protein lysates from procyclic 29–13 (PCF) or single marker bloodform cells (BF) were loaded on an SDS-PAGE and transferred to nitrocellulose. Following detection of CK2 α , the blot was stripped and reprobbed with anti- β -tubulin mAb. Blots were imaged by IR scanning.

Figure 8. Effect of CK2 depletion on cell growth

A. Effect on growth for depletion for both CK2 α and CK2 α' . Growth of procyclic 29–13 cells harboring an inducible RNAi construct targeting the degradation of both CK2 α and CK2 α' RNA was monitored after induction. Shown is the cumulative cell count for both induced (Tet+) and uninduced (Tet-) cultures.

B. Northern analysis. RNA was extracted from either induced (Tet+) or uninduced (Tet-) cells at either day 2 or day 7 post induction. Northern blots were probed concurrently for CK2 α and CK2 α' . The location of the transcripts is indicated along with the double-stranded RNA (ds). Below the figure is shown the relative level of abundance of the transcripts after induction of RNAi as normalized to α -tubulin.

Table 1

Primers

Name	Restriction Site	Sequence 5' > 3'
P-CK2 α 1	<i>Hind</i> III	CCCAAGCTTATGTCCGTCTCTTCGCAACCTAAC
P-CK2 α 2	<i>Bam</i> HI	CGGGATCCCTCCGTGACAACATTCCCTCTTTTCTTCTC
P-CK2 α 3	<i>Hind</i> III	AATGGCTGAAGCTTATGTCCGTCTCTTCGCAACCTAACACA
P-CK2 α 4	<i>Xho</i> I	AATTGCCCTCGAGCACATCACGATGCATTATTCC
P-CK2 α '1	<i>Bam</i> HI	ACTATATTGAATTCCGGATCATATCATGTCCCGCAGCGGTGGTC GC
P-CK2 α '2	<i>Xho</i> I, <i>Xba</i> I	TCCCACCTCGAGCTATCTAGA TCTGCGCTCGGGTACTGTTC
P-CK2 α '3	<i>Xba</i> I, <i>Hind</i> III	GCTCTAGAAAGCTTATGTCCCGCAGCGGTGGTC
P-CK2 α '4	<i>Xho</i> I	AATTCCCTCGAGGTTTCAAGTTGTGCGCCGTCATG
P-CK2 α '5	<i>Xho</i> I	AATTCCCTCGAGGATGTGAGACTACCGCTGTTT
P-CK2 β 1	<i>Eco</i> RI, <i>Bam</i> HI, <i>Eco</i> RV	ACTATATTGAATTCCGGATCCGATATC ATGAAC TACCCGATGTATGTTG
P-CK2 β 2	<i>Xho</i> I, <i>Xba</i> I	TCCCACATCCTCGAGCTATCTAGA TCTCTTTGGTGTCTCCTCAG
CK2 α -RT F		CCTCCACAAGTACACCTAACACT
CK2 α -RT R		CGTCCAAGGCTTCTTACCATGTC
CK2 α '-RT F		ATGAGCCGTACGAGCTTCTG
CK2 α '-RT R		CTGCGCGCCCGAAAC
CK2 β -RT F		GGCTGCACAACAAGGAAAAGTG
CK2 β -RT R		GCACCACCACCAACAC
α Tub-RT F		CGCTCGTATTGACCACAAATTCG
α Tub-RT R		ACCCTCACCGACGTACCA
MHTAP S		ATGGAACAGAACTGATCTCTGAAGAAGACCTGCACCATCATCACCACCATCACCATTCTAGAATGGAAAAGAGAAGAT
MHTAP AS		CAGGTCTTCTTCAGAGATCAGTTTCTGTTCCATGGATCCCTCGAGAAGCTTCCTAGGGTGAATTTTACTTTTGGTGTAAT
CK2 α T7 5'		GATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACC ACAACGGTTAGAAGGAGATATACATA7GTCCGTCTCTTCGCAAC CT
CK2 β T7 5'		GATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACC ACAACGGTTAGAAGGAGATATACATA7GAACTACCCGATGTATG TT
CK2 α TnT 3'		CGTTTTTTTTTTGGCTACTCCGTGACAACATTCCCTCT
CK2 β TnT 3'		CGTTTTTTTTTTGGT7ATCTCTTTGGTGTCTCCTCAGG
ScNOG1 f1		AATTCCAGCTGACCACCATGTCCACAATCTACAATTTCAAAACG GTG
ScNOG1 r1		GATCCCGGGGAATTGCCATGTGTCAGCGTCTGTCGCGTCTGCCGTT AG
ScNOG1 f2		CTATCTATTTCGATGATGAAGATACCCACCAAACCCAAAAAAG AGATCGAATTC CAGCTGACCACCATG
ScNOG1 r2		CTTGCGGGGTTTTTCAGTATCTACGATTCATAGATCTCTGCAGG TCGACGGATCCCGGGGAATTGCCATG

Restriction sites used are underlined and the start and stop codons are italicized