A Novel Protein Kinase CK2 Substrate Indicates CK2 Is Not Directly Stimulated by Polyamines in Vivo

Kathryn Lawson, Laura Larentowicz, Stephen Artim, Candace S. Hayes, and Susan K. Gilmour*

Lankenau Institute for Medical Research, 100 Lancaster Avenue, Wynnewood, Pennsylvania 19096

Received December 5, 2005

ABSTRACT: The activity of the protein kinase (CK2) is enhanced in vitro by the binding of polyamines to the CK2β regulatory subunit. The overexpression of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, also elevates CK2 kinase activity in primary keratinocytes and tissues of K6/ODC transgenic mice. In an effort to better characterize the mechanisms by which polyamines may affect CK2 in vivo, we constructed a transfectable CK2 substrate cDNA consisting of the enhanced green fluorescence protein appended with a canonical CK2 phosphorylation sequence (EGFP-S). In contrast to unmodified EGFP, the EGFP-S protein was extensively phosphorylated by CK2, and this phosphorylation was stimulated by the polyamine spermine in a dose-dependent manner. The in vivo phosphorylation of EGFP-S was examined in cell lines which inducibly express either wild-type CK2 holoenzyme or a CK2 holoenzyme which contains activating mutations in the polyamine-binding region of its CK2β regulatory subunit. Neither the overexpression of ODC in either cell line nor the mutation of the CK2β subunit conferred an increase in CK2 kinase activity as measured by the in vivo phosphorylation of EGFP-S. Rather, our data indicate that polyamines increase total CK2 kinase activity through increases in steady-state levels of both CK2α and CK2β subunits. The overexpression of ODC resulted in a 3-fold increase in steady-state levels of both exogenous and endogenous CK2 transcripts but did not increase the half-life of wild-type or mutated CK2 protein. These data suggest that the regulation of intracellular CK2 by the polyamines may occur through mechanisms distinct from the direct stimulation of CK2 by polyamines in vitro as previously described.

Protein kinase CK2 is a pleiotropic serine/threonine protein kinase present in the cell cytoplasm and nucleus of all eukaryotic organisms (*1*). The CK2 tetrameric holoenzyme is composed of two and sometimes three dissimilar subunits: two α or α' subunits of 38−44 kDa and two β subunits of 24−29 kDa. CK2 holoenzyme formation occurs through the initial dimerization of two β subunits and then subsequent recruitment of α or α' catalytic subunits into the complex (*2*), resulting in the formation of $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ tetramers (*2*, *3*). The regulatory β subunit has been shown to confer stability on the holoenzyme and also modulates both the catalytic activity (*4*) and substrate specificity of CK2α. To date, over 300 substrates of CK2 have been identified (*5*). CK2 phosphorylation functionally modulates the activities of proteins involved in a variety of biological processes, including gene expression, transformation, and apoptosis (*5*). A critical role for CK2 in cell viability is supported by the lethality of CK2α knockouts in yeast (*6*). In mammalian cells, microinjection of antisense oligonucleotides or antibodies against either the α or β subunits of CK2 has been shown to cause mitotic arrest (*7*, *8*).

CK2 activity is elevated in proliferating cells, and abnormally high levels of CK2 have been observed in many human cancers (*9*−*12*). CK2 phosphorylates and modulates the activity of a number of proto-oncogenes and tumor suppressors, including c-myc (*13*), p53 (*14*), and BRCA1 (*15*). Overexpression of the CK2α catalytic subunit has been shown to transform lymphocytes of transgenic mice and accelerate the development of thymic lymphomas in mice lacking p53 (*16*). Recent studies have demonstrated that CK2 also plays a role in apoptosis. CK2 phosphorylation prevents the Caspase-mediated cleavage of several proapoptotic proteins (*17*−*20*), and depletion of CK2 has been shown to either induce apoptosis directly or sensitize tumor cells to apoptosis induction (*21*−*24*), a strategy which is currently under investigation as a potential means of antitumor therapy (*21*, *25*). These studies suggest that CK2 levels can dramatically impact both cell survival and tumorigenesis and underscore the need to elucidate those mechanisms by which CK2 activity is itself modulated in vivo.

Although the precise mechanisms which modulate intracellular CK2 activity still remain unclear, one group of effector molecules that have been shown to modulate CK2 activity is the polyamines. These inorganic polycations are indispensable for cell proliferation and differentiation (*26*, *27*) and have been shown to stimulate the kinase activity of isolated CK2 holoenzyme via their interactions with the CK2β regulatory subunit. Polyamine synthesis is regulated by the rate-limiting enzyme ornithine decarboxylase (ODC), which catalyzes the synthesis of the diamine putrescine, a precursor molecule to the longer chain polyamines, spermidine and spermine. Like CK2, ODC activity and polyamine levels are elevated in proliferating cells and tumors and can

* To whom correspondence and requests for reprints should be addressed. Telephone: (610) 645-8429. Fax: (610) 645-2205. E-mail: gilmours@mlhs.org.

10.1021/bi052480i CCC: $33.50
Published on Web 01/13/2006

be upregulated by many of the same mitogenic stimuli (*28*, *29*).

Affinity labeling studies with photactivatable spermine analogues have revealed that the CK2β regulatory subunit contains a polyamine binding domain which is localized to an acidic stretch between amino acids 55 and 80 (*30*). Electrostatic interactions between polyamines and several key glutamic acid residues in this region (Glu 60, 61, 63) have been found to be particularly important for the polyamine-dependent stimulation of CK2 (*31*). Several studies indicate that the CK2β acidic loop containing these residues normally makes contact with a basic cluster on the CK2α subunit, exhibiting an intrinsic negative regulation on CK2 acitivity that is relieved upon polyamine binding and charge neutralization (*32*, *33*). The affinity of the polyamines for CK2 and their effect on its activity directly correlate with the degree of positive charge. The tetraamine spermine exerts the strongest binding and activation of CK2 whereas the diamine putrescine exhibits the least (*31*). Polyamines have also been shown to negatively affect the phosphorylation of another class of physiological CK2 substrates (notably calmodulin) by displacing polybasic activators such as polylysine or histones which compete for binding at the same residues (*32*, *34*). The electrostatic neutralization which occurs between either class of effector molecule and the CK2β subunit can be mimicked by alanine substitution mutations at the relevant glutamic acid residues. These mutations confer a basal kinase activity toward both classes of substrates which is three to four times that of the wild-type holoenzyme (*35*).

The autoinhibitory effect of the CK2β acidic loop on CK2α activity can be partially overcome by an increase in ionic strength. Polyamine-dependent CK2 activation at physiological sodium or magnesium concentrations is reduced as the concentration of these salts increases (*36*), and a high concentrations of either can stimulate CK2 activity in the absence of polyamines (*36*, *37*). The CK2β subunit contains a high- and a low-affinity magnesium binding site, the latter of which can compete with polyamines for binding (*36*). Similarly, the decreased responsiveness of CK2 to polyamines at higher sodium levels suggests that these molecules may affect CK2 through overlapping mechanisms. These findings have lent support to the idea that because normal physiological concentrations of sodium and magnesium levels are suboptimal for maximal CK2 activity, the polyamines may act as additional polycationic effectors in vivo to confer maximal activity to the enzyme in a low ionic strength environment.

We have previously demonstrated that CK2 kinase activity is increased in both ODC-overexpressing keratinocytes and in the skin of K6/ODC transgenic mice (*38*). The abrogation of this response by the ODC inhibitor α-difluoromethylornithine (DFMO) is in agreement with other studies demonstrating a similar downregulation of CK2 activity in DFMO-treated adult human keratinocytes (*39*) as well as xenotransplanted colorectal neoplasias (*40*). We also observed an increased CK2 kinase activity in ODC-overexpressing cells that appeared to be disconcordant with static levels of CK2 protein in the cytosol (*38*). Given that CK2 has been shown to be activated by direct binding of the polyamines in vitro, our data suggested that this may be a regulatory mechanism for the intracellular regulation of CK2 as well.

In our present work, we examined the mechanism(s) by which the polyamines increase CK2 kinase activity in vivo, focusing particularly on the direct stimulation of CK2 activity by interaction of the polyamines with the CK2β subunit. We examined the effect of increased polyamine levels on the activity of epitope-tagged CK2 tetramers containing wild-type CK2β or a CK2β subunit in which activating glutamic acid mutations (mimicking polyamine binding to CK2β) had been introduced. The activity of wild-type CK2 tetramers isolated from normal and ODC-overexpressing cells was compared to determine their activities in isolated complexes. We also examined the in vivo phosphorylation of a novel EGFP-CK2 substrate reporter protein in cells expressing either polyamine-stimulated wild-type or mutant CK2. The overexpression of ODC did not result in hyperactive CK2 tetramers within the intracellular environment, nor did CK2β mutations, which stimulate CK2 activity in vitro, increase the in vivo phosphorylation of the CK2 substrate reporter. Steady-state levels of both CK2 mRNA and protein were elevated in ODC-overexpressing cells, suggesting that polyamine-stimulated increases in CK2 activity in vivo are mediated at the level of gene expression and are not the result of a direct interaction with the CK2β subunit, as had been previously demonstrated in vitro.

MATERIALS AND METHODS

Cell Culture. Ecr293 cells (Invitrogen) stably transfected with the construct pVgRxR were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 400 μg/mL zeocin (Invitrogen). For expression of CK2 holoenzyme, Ecr293 cells were transfected with the constructs pIND-CK2β^{WT}/α or pIND-CK2β^{EA}/α using Lipofectamine 2000 (Invitrogen) and selected with 400 μg/mL G418 (Invitrogen). Clonally selected cells were screened for inducible CK2 expression by treating the cells for 24 h with either the inducing agent, 10 μM ponasterone (Invitrogen), or an equivalent volume of ethanol vehicle and quantitating the level of Flag-tagged CK2β protein in these lysates by immunoblot analyses.

pCXODC and pCXEGFP or pCXEGFP-S transfections were performed by Lipofectamine-mediated transfection of these constructs into clonally selected Ecr293 cells which expressed an ecdysone-inducible CK2β^{WT}/α or CK2β^{EA}/α holoenzyme. Ponasterone was added to the cells 4 h after transfection, and cells were analyzed as indicated after 24 h of ponasterone treatment. For radiolabeling with [^{32}P]-orthophosphate, cells were then rinsed three times with phosphate-free DMEM (Biosource) and incubated with the same medium for 1 h. After 1 h, the prelabel medium was aspirated and fresh phosphate-free DMEM supplemented with [^{32}P]orthophosphate (300 μCi/mL) was added to the cells, which were further incubated for 2 h at 37 °C. At the end of the radiolabeling period, cells were again washed three times with phosphate-free DMEM, and total soluble extracts were prepared as described. Ponasterone was present at 10 μM in both the prelabeling and labeling media.

Half-life studies were performed by treating cells with 10 μM ponasterone for 24 h and then replacing the medium with DMEM/10% FBS supplemented with 10 μg/mL cycloheximide. Extracts were prepared as described at the time of cycloheximide administration and the indicated time points.

Cloning of cDNAs. The construct pIND-IRES was generated by digestion of the IRES sequence in pIRES (Clontech) with NheI and XbaI restriction enzymes (Promega) and ligating into the corresponding multiple cloning sites in the ecdysone-inducible vector pIND (Invitrogen). cDNAs of human CK2α and CK2β (a kind gift from H. Meisner) were PCR amplified using the Platinum Taq polymerase (Invitrogen). Recombinant PCR was used to introduce glutamic acid to alanine substitution mutations at codons 60, 61, 63, 73, and 77 of the human CK2β cDNA (denoted CK2β^{EA}). Both wild-type and mutant CK2β cDNAs were amplified using the 5′ primer CACCGTCGCTAGCAGATCTTCGCCACCATGGACTACAAAGACGATGACGACAAGATGAGCAGCTCAGAGGAGGTG and the 3′ primer CCTATCCACGCTAGCTCAGCGAATCGTCTTGACTGG, which introduced a Flag epitope at the 5′ end of CK2β. The CK2α cDNA was amplified using the 5′ primer AGTAGTGGAGCTAGCATGGGGGGTTCTCATCATCATCATCATCATGGTATGTCGGGACCCGTGCCAAGC and the 3′ primer CTGCAGTCAGCTAGCGGATCCTCAGGAGACAGATAGGGCCGT, which added a 6×-His epitope to the 5′ end of the CK2α sequence as well as *Nhe*I restriction sites at both ends. *Nhe*I-cut CK2α was ligated into the *Xba*I site in the second multiple cloning site of pIND-IRES. *Nhe*I restriction sites engineered into the CK2β primers were used to ligate the modified wild-type or mutant CK2β sequences into the first multiple cloning site of pIND.

The EGFP-S CK2 substrate fusion protein was created by PCR amplification of the enhanced green fluorescence protein from the vector pEGFP-C1 using the forward primer CCGCGAATTCTGCAGGTCGCCACCATGGTGAGCAAG and the reverse primers AGTGAATTC TTAGTCGTC GTCGCTGTC GTCGTCCCT CCTCCTCTT GTACAGCTC GTCCATGCC which appended the amino acid sequence RRRDDDSDDD to full-length EGFP. The pCXODC construct was created by PCR cloning of a murine ODC cDNA (*41*) into the *Eco*RI site of the plasmid pCXEGFP, derived from the plasmid pCAG (*42*). All PCR products were sequenced to ensure the integrity of the cDNAs.

Extract Preparation. For total soluble cell lysates, cells were rinsed twice with PBS and lysed in Tween buffer (50 mM Tris-HCl, pH 7.4, 0.1% Tween 20, 1 mM dithiothreitol) supplemented with 2 μg/mL freshly added aprotinin, leupeptin, and pepstatin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 0.4 mM Pefabloc (Roche Diagnostics). Extracts were further homogenized by passing through a syringe needle after a 60 min incubation on ice. Extracts were centrifuged at 14000*g* at 4 °C for 15 min, and the supernatant was collected as the soluble fraction. For ODC analysis, cells were rinsed twice in PBS, lysed in ODC assay buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 μg/mL aprotinin, leupeptin, and pepstatin, 0.2 mM PMSF, and 1 mM NaF). ODC activity was assayed as described previously (*38*, *41*, *43*). Polyamine levels were determined as previously described (*41*, *43*) by HPLC analysis of the dansylated products after overnight extraction with 0.2 N perchloric acid.

Immunoprecipitation. To immunoprecipitate epitope-tagged CK2 or EGFP-derived proteins, total soluble cell extracts were diluted to 0.25 mg of protein/mL in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 0.1% Tween 20, 1 mM DTT, 2 μg/mL aprotinin, leupeptin, and pepstatin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 0.4 mM Pefabloc. Lysates were precleared with a 20 μL packed bead volume of protein A or protein G agarose (Sigma) for 30 min. The lysates were then centrifuged, and the supernatant fraction was transferred to a new tube and incubated for 60 min with a 6×-His mouse monoclonal (Labvision), an EGFP rabbit polyclonal (Clontech), or an agarose-conjugated anti-Flag antibody (Sigma). Lysates incubated with nonconjugated primary antibodies were further incubated with protein A (rabbit) or protein G (mouse) agarose beads for an additional 60 min. The beads were washed five times in IP wash buffer (50 mM Tris-HCl, pH 7.4, 0.1% Tween 20, 500 mM NaCl, 2 μg/mL aprotinin, leupeptin, and pepstatin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 0.4 mM Pefabloc). In experiments where recovery of polyamine-bound CK2 was critical, NaCl was omitted from the wash buffer. CK2 complexes were immediately used in a casein phosphorylation assay. After the last wash in IP buffer, EGFP immunocomplexes were washed twice in either CK2 assay buffer or λ phosphatase buffer (New England Biolabs) as noted. EGFP immunocomplexes were either used directly in a CK2 phosphorylation assay or dephosphorylated with λ phosphatase (New England Biolabs) prior to phosphorylation with recombinant human CK2 (Calbiochem).

Immune Complex Kinase Assays. (A) Immunoprecipitated CK2. CK2 holoenzyme was immunoprecipitated with Flag- or His-directed antibodies as described. After the final wash step, complexes were washed twice in CK2 assay buffer (10 mM Tris-HCl, pH 7.4), and radioactive phospholabeling of casein substrate was initiated by the addition of radioactive assay mix (10 mM Tris-HCl, pH 7.4, 0.3 μg/μL casein, 1 mM $MgCl_2$, 50 μM ATP, and 6 μCi of [γ-^{32}P]ATP) in a final volume of 50 μL. After 5 min, the kinase reaction was stopped by the addition of 10 μL of 6× SDS−PAGE sample buffer (*44*) and boiled for 5 min prior to loading on an SDS−PAGE gel. For polyamine-dependent CK2 kinase reactions, the recovered immunocomplexes were divided into equal aliquots and preincubated with either CK2 assay buffer or CK2 assay buffer supplemented with 600 μM spermine for 30 min prior to the addition of the radioactive assay mix (300 μM spermine final concentration). The total reaction volume was kept at 50 μL in all cases.

(B) Immunoprecipitated EGFP-S CK2 Substrate. EGFP or EGFP-S fusion protein was immunoprecipitated with anti-GFP antibodies as described. After the final wash step, complexes were washed twice in either CK2 reaction buffer or λ phosphatase assay buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.01% Brij 35, and 1 mM DTT). Immune complexes that were dephosphorylated prior to CK2 phosphorylation were then incubated with 1000 units of λ phosphatase enzyme (New England Biolabs) in λ phosphatase buffer supplemented with 2 mM $MnCl_2$ for 30 min at 34 °C. After dephosphorylation, immune complexes were washed twice in CK2 assay buffer supplemented with 2 mM sodium fluoride and sodium orthovanadate. CK2 phosphorylation was carried out under the conditions described above, using 20 units of recombinant human CK2 (Calbiochem) per reaction. For polyamine-dependent EGFP phosphorylation analyses, the recovered immunocomplexes were equally divided into three aliquots and subjected to phosphorylation in reactions supplemented with either 0, 100, or 500 μM

spermine. After 5 min, the kinase reaction was stopped by the addition of 10 μL of 6× SDS−PAGE sample buffer (*44*) and boiled for 5 min prior to analysis by SDS−PAGE, immunoblotting, and autoradiography.

Immunoblotting and Autoradiography. Proteins from total cell lysates or the supernatant fraction from boiled immunocomplexes were separated by SDS−PAGE and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S (Sigma) to verify that equal levels of casein or EGFP were present in CK2 phosphorylation reactions. Membranes with radiolabeled protein were first dried and exposed to a phosphor-sensitive screen for quantitative analysis of autoradiographic data by the PhosphorImager system (Molecular Dynamics). After collection of autoradiographic images, membranes were rewetted and blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) and then probed with primary antibodies diluted in 0.1% Tween/TBS. Antibodies used included anti-Flag M2 mouse monoclonal (Sigma), anti-His rabbit polyclonal (Labvision), or anti-EGFP rabbit polyclonal (Clontech). Immunoblots were developed using fluorochrome-conjugated secondary antibodies (Rockland Immunochemicals; Molecular Probes) in conjunction with the Odyssey infrared imaging system (LI-COR). Relative band intensities were quantitated using Odyssey-based software analysis of collected images.

RT-PCR Analysis. Total RNA was isolated from ponasterone-treated, mock- or ODC-transfected cells using TriReagent (Molecular Research Center, Cincinnati, OH). Two micrograms of total RNA was reverse-transcribed using Super Script II RNase H reverse transcriptase (Life Technologies, Gaithersburg, MD) and random hexamer primer (Amersham Pharmacia Biotech) at 42 °C for 1 h for cDNA synthesis. The reverse transcription product was diluted 15-fold in water, and 10 μL of template was used for PCR. PCR reactions were performed in a 50 μL volume consisting of 10 μL of template and 40 μL of PCR master mix, which included Red Pol polymerase (Gene Choice, Frederick, MD), nucleotides, and primers for either the exogenous CK2 transcript or endogenous transcripts of CK2α or CK2β. Each master mix was also supplemented with 3′-^{32}P-end-labeled primers for both GAPDH and the CK2 transcript of interest. The reactions consisted of a 2 min initial denaturation (95 °C) followed by 30 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 1 min) followed by a final elongation step of 5 min at 72 °C. Twenty microliters of PCR products was electrophoresed on 2% agarose gels, which were then dried and exposed to a phosphor-sensitive screen for quantitative analysis by the PhosphorImager system (Molecular Dynamics). Primers against the human CK2α or CK2β mRNAs were designed to span intron−exon borders in order to distinguish RTPCR products from those of contaminating genomic DNA. A constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control. The specific primer pairs used were as follows: for GAPDH (600 bp product), forward primer 5′-TGCTGAGTATGTCGTGGAGTC-3′ and reverse primer 5′-AGTGGGAGTTGCTGTTGAAGT-3′; for CK2α (315 bp product), forward primer 5′-CATGAGCACAGAAAGCTACG-3′ and reverse primer 5′-AGCGTTCCCATCGCTTTCGA-3′; for CK2β (434 bp product), forward primer 5′-CAGGTCCCTCACTACCGACA-3′and reverse primer 5′-CAGCTGGTAGGCCATCGGAT-3′; for exogenous CK2β/CK2α single transcript (392 bp product), forward primer 5′-GCTACATCCTTACCAACCGT-3′ and reverse primer 5′-CCCTAGATGCATGCTCGACG-3′.

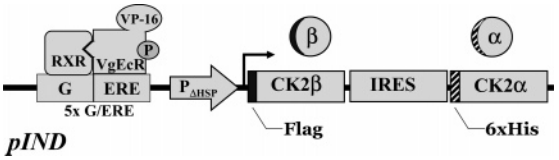


FIGURE 1: Ecdysone-inducible expression of wild-type and mutant CK2 holoenzyme. Wild-type or mutant CK2β subunits were coexpressed with wild-type CK2α through the use of an incorporated internal ribosome entry site (IRES) sequence, which enabled translation of CK2β and CK2α subunits from a single ecdysone-inducible transcript. CK2β and CK2α cDNAs were modified to express a Flag and 6×-His epitope at their respective carboxy termini and introduced into the ecdysone-inducible plasmid pIND. Each CK2β/CK2α pIND construct was transfected into cells which stably expressed the transcription factors RXR and an ecdysone-responsive VgECR-VP16 fusion protein (Ecr293, Invitrogen). Upon administration of the ecdysone analogue ponasterone (P), these transcription factors bound to five copies of a hybrid glucocorticoid-ecdysone response element (G/ERE), stimulating transcription from a minimal heat shock promoter ($P_{\Delta HSP}$) and thus enabling the expression of both CK2 subunits simultaneously.

RESULTS

Ecdysone-Inducible CK2 Constructs. To better characterize the in vivo effects of the polyamines on the CK2 holoenzyme, stably transfected cell lines were created which inducibly expressed both a CK2α catalytic subunit and either a wild-type or mutant version of the polyamine-responsive CK2β regulatory subunit (Figure 1). Utilization of an ecdysone-inducible promoter allowed the establishment of stable clones and circumvented the problem of CK2 down-regulation seen in stable transfectants when exogenous CK2 expression was constituitively regulated (personal observation). Several key glutamic acid residues of CK2β have been previously implicated in the interaction between this subunit and the polyamines. The substitution of Glu 60, Glu 61, and Glu 63 to alanine conferred an elevated basal activity to the holoenzyme that was 4-fold that of wild-type CK2 (*35*). These substituted residues have been shown to induce a conformational change in the holoenzyme which constitutively mimics that which occurs upon binding of polyamines to the CK2β subunit. On the basis of identifiable interactions between CK2β and a photoactivatable spermine analogue, Glu 73 and Glu 77 have also been predicted to be likely candidate residues for spermine binding (*30*). To determine whether such mutations were able to affect the kinase activity of CK2 in vivo, a CK2β mutant (CK2β^{EA}) cDNA containing alanine substitution mutations at all five residues (60, 61, 63, 73, and 77) was stably transfected into cells under the control of an ecdysone-inducible promoter. The CK2β and CK2α proteins were tagged with Flag and 6×-His epitope, respectively.

Inducible Expression of CK2 α and β Subunits. HEK293 cells previously transfected with the VgRXR construct were transfected with the inducible CK2 holoenzyme constructs and subjected to selection. Several clones were isolated which displayed tightly regulated, highly inducible CK2 expression under the control of ponasterone, as analyzed by expression of the exogenously expressed CK2β-Flag (Figure 2A).

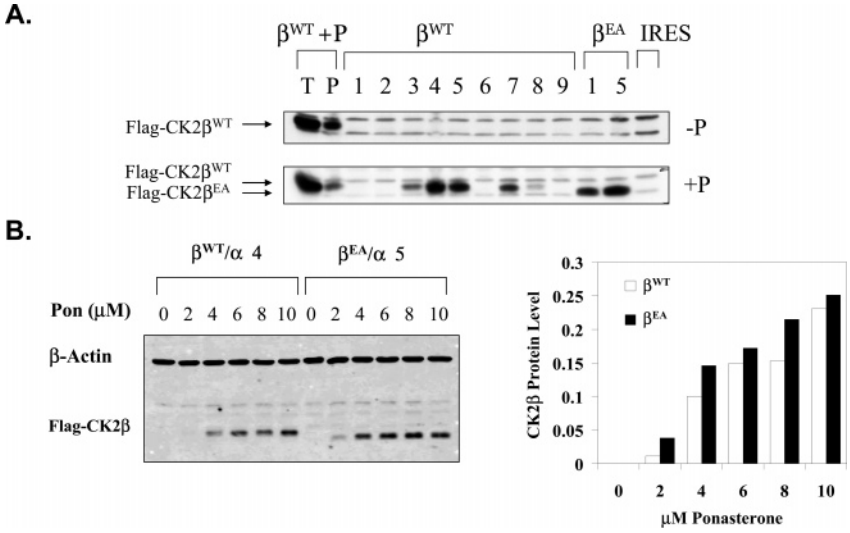


FIGURE 2: Inducible expression of CK2 in clonally selected cells. (A) Immunoblotting of CK2β-Flag in EcR293 stably selected clones. EcR293 cells were stably transfected with pIND-CK2β^{WT}/α or pIND-CK2β^{EA}/α and screened for inducible expression of Flag-tagged CK2β subunits. Each clonal population was treated with (+Pon) or without (−Pon) 10 μM ponasterone for 24 h prior to harvest. Lysates from ponasterone-treated, transiently transfected cells (T) and pooled clones (P) were used as a positive control for CK2β-Flag detection. Lysates from cells transfected with empty pIND vector (IRES) were used as a negative control. β^{WT}:1−9 and β^{EA}:1,5 refer to individual clone designations. (B) Ponasterone dose−response of CK2β^{WT}/α and CK2β^{EA}/α transfected stable clones. CK2β^{WT}/α clone 4 (□) or CK2β^{EA}/α clone 5 (■) were treated for 24 h with 0, 2, 4, 6, 8, and 10 μM ponasterone prior to harvest. Equal amounts of protein were analyzed by immunoblot for Flag-CK2β expression. β-Actin levels were used as a normalization control. Relative levels of Flag-CK2β were quantitated for each cell line by normalizing to β-actin.

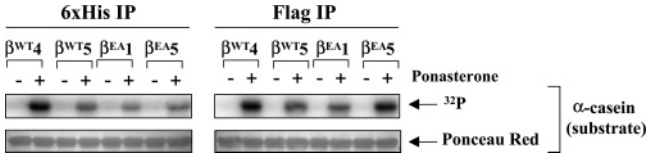


FIGURE 3: Incorporation of CK2α-His and CK2β-Flag into active CK2 complexes. Ecr293 clones which inducibly expressed His-tagged CK2α and Flag-tagged CK2β were incubated with or without 10 μM ponasterone for 24 h prior to lysis. CK2 was immunoprecipitated from each lysate using antibodies directed against the 6×-His or Flag epitopes. Kinase activity of each immunocompex was assayed by incubating each complex with [γ-^{32}P]ATP and dephosphorylated α-casein. The reaction mixes were then subjected to SDS−PAGE, blotted onto nitrocellulose, and analyzed by autoradiography. Ponceau red staining of the nitrocellulose was used to confirm equal quantities of the casein substrate.

CK2β^{WT}/α clones 4 and 5 and CK2β^{EA}/α clones 1 and 5 were used for subsequent studies. Ponasterone titration of both CK2β^{WT}/α and CK2β^{EA}/α clones was performed to determine optimal concentrations which would yield similar levels of expression (Figure 2B). Ponasterone at 10 μM gave maximal induction of both proteins and similar levels of expression and was used for all further studies.

Kinase Activity of Exogenously Expressed CK2 Holoenzyme. Incorporation of both His-CK2α and Flag-CK2β subunits into active CK2 complexes was verified by immunoprecipitation of these proteins with antibodies directed against their respective epitope tags (Figure 3). CK2 kinase activity of the immunocomplexes was then analyzed by a casein phosphorylation assay. Immunoprecipitation of His-CK2α or Flag-CK2β yielded active CK2 complexes in only lysates from induced cells, again indicating the tight regulation of exogenous CK2 expression. Antibodies directed against the 6×-His tag immunoprecipitated active CK2 complexes, indicating that the 6×-His tag does not interfere with the kinase activity of the active subunit. The incorporation of the exogenously expressed Flag-CK2β regulatory subunit into the CK2 holoenzyme was demonstrated by the associated CK2 kinase activity of anti-Flag immunocomplexes.

Due to its bulky nature, casein was utilized as a substrate in preference to more specific CK2 substrates, as we and others have noted that polyamine-dependent phosphorylation of smaller molecules such as the canonical CK2 peptide substrate RRRDDDSDDD did not occur at physiological magnesium concentrations (*31*). It is unlikely, however, that the kinase activity shown here is due to a kinase other than CK2, as the exogenously expressed CK2 subunits in these experiments were immunoprecipitated with epitope-specific antibodies. Furthermore, this activity was only detectable in immunoprecipitated material from ponasterone-induced, transfected cells. No kinase activity was detected in immunoprecipitated material from ponasterone-treated parental cells or from uninduced transfectants (data not shown).

Polyamine Stimulation of Exogenous CK2 Holoenzyme. It has been previously demonstrated that binding of the polyamines to the polyamine binding site of CK2β induces a conformational change in the CK2 holoenzyme and increases its kinase activity (*31*). The ability of polyamines to increase the kinase activity of epitope-tagged CK2β^{WT}/α and CK2β^{EA}/α holoenzyme complexes was examined by performing immune complex kinase assays in the presence or absence of 300 μM spermine (Figure 4). Anti-Flag antibodies were used to ensure that the immunoprecipitated holoenzyme complex contained at least one of the CK2β subunits of interest, since CK2β dimerization is a prerequisite for proper assembly of the holoenzyme tetramer (*45*). The phosphorylation of casein from these assays was quantitated by phosphorimager analysis and normalized to the amount of immunoprecipitated CK2 present in each reaction. CK2β^{EA}/α complexes exhibited a higher basal level of kinase activity than did CK2β^{WT}/α, and both forms of CK2 were

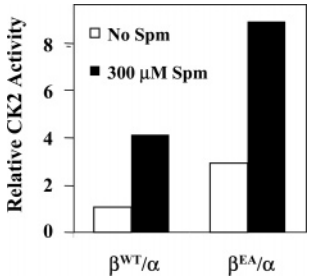


FIGURE 4: Effect of polyamines on the phosphorylation of casein by epitope-tagged CK2 holoenzyme. Ecr293 parental cells were transiently transfected with either pIND-CK2β^{WT}/α (β^{WT}/α) or pIND-CK2β^{EA}/α (β^{EA}/α) constructs and treated with 10 μM ponasterone for 24 h prior to harvest. Anti-Flag immune complexes from each lysate were divided into two equal fractions, and each fraction was incubated in the absence (□) or presence (■) of 300 μM spermine for 30 min prior to kinase assay. Phosphorylation of casein substrate by the immunocomplexes was analyzed by autoradiography, and relative phosphorylation values were normalized to densitometric analysis of anti-Flag immunoblots of the same membrane.

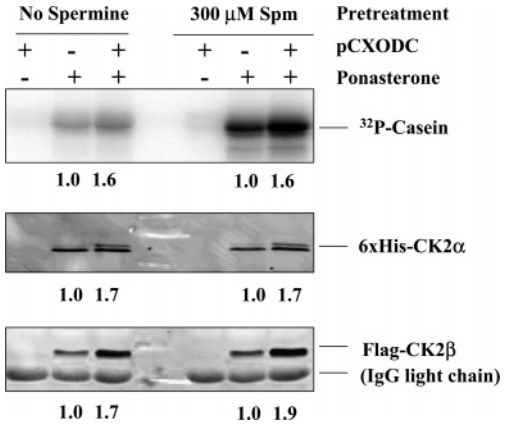


FIGURE 5: Polyamine-dependent activation of CK2 complexes immunoprecipitated from mock- and ODC-transfected cells. CK2β^{WT}/α inducible cells (clone 4) were transfected with pCXODC or empty vector control and induced with ponasterone prior to lysis. pCXODC cells not treated with ponasterone were used as a negative control. Immunoprecipitated anti-Flag-CK2 complexes from each cell lysate were split into two equal fractions and incubated in CK2 assay buffer supplemented with 0 or 300 μM spermine. Kinase activity of each immunocomplex was then analyzed by casein phosphorylation assay. Relative levels of exogenous CK2 subunits present in each kinase reaction were determined by anti-His and anti-Flag immunoblot of the same membrane used for autoradiography. Quantitation of the immunoblots was performed using Odyssey analysis software.

stimulated by spermine. These data indicate that the presence of the epitope tags do not affect the polyamine-mediated stimulation of exogenously expressed CK2. The stimulation of CK2β^{EA}/α by spermine is in agreement with previous studies in which CK2 holoenzymes containing similar mutations of the CK2β subunit displayed an increased basal kinase activity that was further increased upon addition of spermine (*31*).

Effect of ODC Overexpression on Kinase Activity of Immunoprecipitated CK2 Holoenzyme. We have previously demonstrated that CK2 kinase activity is elevated in both ODC-overexpressing keratinocytes and in the skin of ODC transgenic mice (*38*). It is unknown, however, whether the elevation of CK2 kinase activity in ODC overexpressing cells is the result of the interaction of polyamines with the CK2β regulatory subunit. This elevation may also be an indirect result of polyamine-driven signaling cascades, since recent in vitro studies suggest that CK2 basal activity may be increased by its interaction with activated signal transduction proteins (*46*) or via its own phosphorylation on tyrosine residues by other kinases (*47*). To address this question in more detail, we examined the kinase activity of immunoprecipitated CK2 from CK2β^{WT}/α cells expressing normal or elevated levels of ODC (Figure 5). We overexpressed a truncated form of ODC which exhibits the same enzymatic activity as wild-type ODC but is considerably more stable (*48*), resulting in high levels of both ODC activity and polyamine synthesis. pIND-CK2β^{WT}/α stable cell lines were transiently transfected with either control vector or ODC, and exogenous CK2 expression was induced with ponasterone. The kinase activity of anti-Flag immunocomplexes was analyzed by casein phosphorylation assay. To determine if polyamines were able to stimulate CK2 from ODC-overexpressing cells to the same degree as CK2 from normal cells, part of each immunocomplex was preincubated with 300 μM spermine prior to casein phosphorylation. This concentration of spermine has been previously shown to maximally stimulate kinase activity of immunoprecipitated CK2 (*49*).

CK2 activity of complexes from ODC-overexpressing cells showed a 1.6-fold increase in kinase activity compared to ponasterone-treated mock-transfected cells. Quantitation of the Flag-CK2β and His-CK2α subunits recovered in these complexes, however, reveals a similar increase (1.7-fold) in protein levels between ODC- and mock-transfected cells. These data suggest that the increased CK2 kinase activity seen in immunocomplexes from ODC cells is the result of increased levels of recovered CK2 protein. Furthermore, when these values were analyzed by normalizing levels of spermine-stimulated CK2 activity to those of basal activity with respect to each source of CK2, spermine stimulated the activity of CK2 from mock- and ODC-transfected cells to the same extent (about 6.5-fold). This direct correlation between spermine stimulation of activity with CK2 protein level suggests that polyamines are not differentially affecting the activity of the CK2 holoenzyme isolated from ODC-overexpressing cells.

Characterization of a Recombinant EGFP Substrate for in Vivo Analysis of CK2 Kinase Activity. A major caveat of the in vitro analysis of polyamine-dependent biological processes is the ample evidence which suggests that polyamines are localized intracellularly to discrete pools (*50*, *51*) that become diffuse upon cell lysis (*52*). Since CK2 itself has been proposed to exist in distinct pools (*53*, *54*), the lysis of cells to examine CK2 activation by polyamines presumably abrogates the compartmentalization of these molecules and does not accurately reflect the true nature of this interaction in vivo. To more accurately assess polyamine-dependent modulation of CK2 in vivo, we developed a plasmid which constituitively expressed a full-length enhanced green fluorescence protein (EGFP) fused to a canonical CK2 consensus sequence (EGFP-S) (Figure 6A). This approach has been used in the characterization and large-scale bacterial expression of EGFP-based protein substrates which contain consensus phosphorylation sites for the kinases Src, c-abl, protein kinase C βII, or protein kinase A (PKA) (*55*).

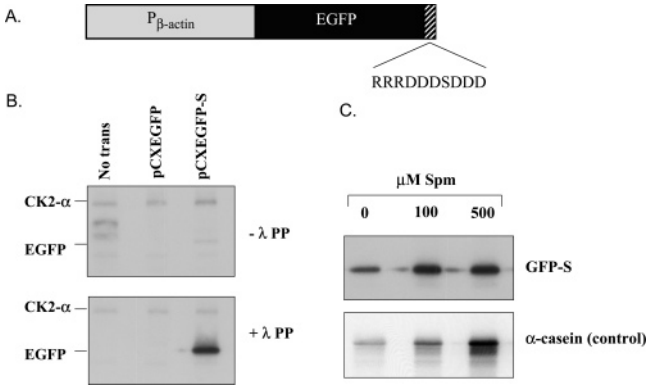


FIGURE 6: Characterization of a novel, artificial in vivo CK2 substrate. (A) Design of EGFP-CK2 substrate. A canonical CK2 phosphorylation sequence was added to the carboxy terminus of full-length enhanced green fluorescence protein (GFP) and constituitively expressed under the control of a chicken β-actin promoter. (B) In vitro phosphorylation of EGFP-CK2 substrates by CK2. Ecr293 cells were transiently transfected with the pCX plasmid expressing either untagged EGFP (pCXEGFP) or full-length EGFP-CK2 substrate (pCXEGFP-S). EGFP protein was immunoprecipitated from each cell lysate with anti-GFP antibodies, and immunocomplexes were incubated in the presence ($+\lambda$) or absence ($-\lambda$) of λ protein phosphatase. After several washes, the complexes were then incubated with [γ-^{32}P]ATP and purified CK2 holoenzyme. The reaction mixes were then analyzed by SDS−PAGE and autoradiography. (C) Polyamine-stimulated phosphorylation of EGFP-CK2 substrate proteins. EGFP-S protein was immunoprecipitated from the same whole cell lysates as in (B) with anti-EGFP antibodies. Immune complexes were divided into equal thirds and phosphorylated with purified CK2 holoenzyme and [γ-^{32}P]ATP in the presence of 0, 100, or 500 μM spermine. Dephosphorylated α-casein was also phosphorylated in the same manner as a positive control for polyamine stimulation of CK2 kinase activity. Phosphorylation of EGFP-CK2 substrate and α-casein was determined by SDS−PAGE and autoradiography. Equal recovery of EGFP proteins for each reaction set was verified by Ponceau staining of nitrocellulose membranes (data not shown).

We examined the ability of recombinant CK2 to phosphorylate unmodified EGFP or EGFP-S immunocomplexes from transiently transfected cells (Figure 6B). Neither the EGFP nor the EGFP-S protein was strongly phosphorylated by CK2 when immunocomplexes were used directly in a CK2 phosphorylation assay. However, the EGFP-S substrate protein was strongly phosphorylated compared to EGFP when the immunocomplexes were preincubated with λ phosphatase prior to the kinase reactions. The requisite dephosphorylation of EGFP-S for efficient phospholabeling by recombinant CK2 in vitro suggests that EGFP-S is also a substrate of endogenous CK2 in EGFP-S transfected cells.

Phosphorylation of EGFP-S by CK2 was further characterized by examining the effects of the polyamines on CK2 phosphorylation of EGFP-S immunocomplexes (Figure 6C). Equal fractions of dephosphorylated EGFP-S immunocomplexes were subjected to phosphorylation by recombinant CK2 in the presence of spermine. Polyamine-dependent stimulation of CK2 toward casein was used as a control for increasing CK2 kinase activity. Phosphorylation of EGFP-S increased with increasing spermine levels. The relative increase in spermine-stimulated EGFP-S phosphorylation was less than that of α-casein, which may be accounted for by the fact that phosphorylation of casein occurs on multiple sites per molecule compared to a single site on EGFP-S.

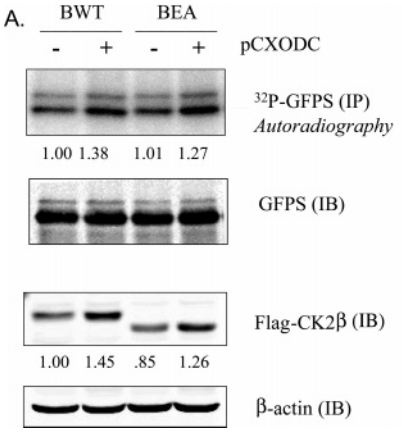
In Vivo Analysis of ODC-Modulated CK2 Kinase Activity Using a EGFP Recombinant Substrate. To better characterize the in vivo effects of the polyamines on CK2 kinase activity, we examined the phospholabeling of EGFP-S in transiently transfected cells (Figure 7). The phosphorylation of the EGFP-S substrate from CK2β^{EA}/α cells was not increased compared to that from CK2β^{WT}/α cells (Figure 7A), indicating that mutations in the polyamine binding region of CK2β are not sufficient to confer increased CK2 kinase activity in vivo. Cotransfection of the pCXODC plasmid increased ODC activity nearly 2 orders of magnitude over that of control transfectants (Figure 7B) and increased EGFP-S phosphorylation in both CK2β^{WT}/α and CK2β^{EA}/α cells. An analysis of Flag-CK2β levels from whole cell lysates revealed that this increase is most likely due to increased levels of CK2 protein and not to an activation of the CK2 holoenzyme with increased polyamines. The phosphorylation of the EGFP-S substrate protein appears to be dependent on the presence of the CK2 substrate sequence, since an analysis of the in vivo phosphorylation of untagged EGFP revealed that EGFP alone is not appreciably phosphorylated in transfected cells with either normal or high levels of ODC activity (data not shown). An analysis of polyamine levels in ODC-overexpressing cells revealed dramatic increases in putrescine, with only moderate changes (2−4-fold) in the levels of the longer chain amines spermidine and spermine (Figure 7C). This pattern of polyamine accumulation is in agreement with our previous analyses of polyamine levels in ODC-overexpressing keratinocytes (*38*).

Effect of ODC on the Stability of Wild-Type and Mutant CK2β Subunits. It has been demonstrated that the autophosphorylation of three key serine residues of the CK2β regulatory subunit protects the protein from ubiquitination and proteasomal degradation (*56*). Since CK2β levels were increased in ODC-overexpressing cells, we used cycloheximide treatment of CK2-overexpressing cells to determine if this increase could be accounted for by polyamine-dependent effects on stability (Figure 8). Mock- or ODC-transfected cells were induced with ponasterone to express wild-type or mutant CK2 tetramers, and then total cell lysates were prepared at various time points after treatment with cycloheximide. Neither the mutation of the CK2β subunit nor the overexpression of ODC conferred increased stability to the CK2β subunit. In fact, we observed a slight reduction in half-life of CK2β (for both wild type and mutant) in cells with elevated levels of polyamines.

Effect of ODC on Steady-State Levels of CK2 mRNAs. To determine if the polyamine-dependent increases in steady-state levels of CK2 were the result of increased transcription, mock- or ODC-transfected cells were induced with ponasterone to express wild-type CK2 holoenzyme. Levels of the exogenous CK2 transcript as well as endogenous CK2α and CK2β transcripts were analyzed by semiquantitative RTPCR (Figure 9). Exogenous CK2α and CK2β transcript levels are representative from a single PCR reaction since they were both expressed from a single transcript. ODC overexpression caused a 3-fold increase in the levels of all CK2 transcripts analyzed, while causing only nominal changes in the GAPDH transcript used as a normalization control.

DISCUSSION

Despite the many reports which have characterized the polyamine-dependent stimulation of CK2 in vitro, the



B.

	ODC Activity
pCX/BWT	0.18 ± 0.10
pCXODC/BWT	106.74 ± 36.07
pCX/BEA	0.11 ± 0.06
pCXODC/BEA	95.13 ± 28.7

C.

	Put	Spd	Spm
Mock	n.d.	10.87	71.65
pCXODC	2679.73	115.00	290.86

FIGURE 7: In vivo CK2 kinase activity of wild-type and mutant CK2 holoenzyme complexes in ODC-overexpressing cells. (A) Phosphorylation of EGFP-S in CK2- and/or ODC-overexpressing cells. pIND-CK2β^{WT}/α and pIND-CK2β^{EA}/α inducible clones were transiently transfected to constituitively express either the EGFP-S CK2 substrate alone or EGFP-S and ODC. Ponasterone-treated, transfected cells were radiolabeled with [^{32}P]orthophosphate, and EGFP-S was immunoprecipitated from total cell lysates. Immunocomplexes were analyzed by SDS−PAGE and autoradiography. Total cell lysates were also analyzed for expression of Flag-CK2β and β-actin. Densitometric analysis was performed by molecular dynamics phosphorimager or Odyssey analysis software. [^{32}P]GFP relative phosphorylation was normalized to recovered protein levels. (B) ODC activity in pCXODC- or mock-transfected CK2-expressing cells from (A). After transfection, each transfectant population was split into two dishes of equal density, and the population not used for radiolabeling was analyzed for ODC activity as described in Materials and Methods. ODC activity is expressed as nanomoles of $^{14}CO_2$ released per hour per milligram of protein. Values shown are the means of duplicate experiments. (C) Polyamine levels in pCXODC- or mock-transfected CK2-expressing cells. pIND-CK2β^{WT}/α cells were transiently transfected with a pCX-derived control plasmid or pCXODC, induced with 10 μM ponasterone, and harvested for polyamine analysis after an additional 24 h. Polyamines are expressed as picomoles per milligram of DNA. Put denotes putrescine, Spd, denotes spermidine, and Spm denotes spermine. Values shown are the means of duplicate experiments.

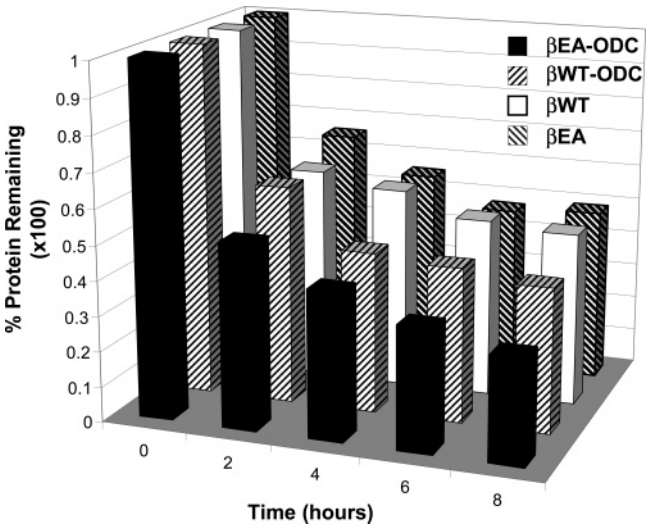


FIGURE 8: Effect of ODC overexpression on steady-state levels of CK2β in cycloheximide-treated cells. pIND-CK2β^{WT}/α (□, ▨) and pIND-CK2β^{EA}/α (▧, ■) clones were transiently transfected with either a pCX-derived control vector (□, ▧) or pCXODC (▨, ■) and induced with 10 μM ponasterone for 24 h. Fresh medium containing ponasterone and 100 μg/mL cycloheximide was added to the cells, and total cell lysates were prepared at 0, 2, 4, 6, and 8 h after cycloheximide administration. Lysates were analyzed by SDS−PAGE and western blot against Flag-tagged CK2β. Protein levels were quantitated and normalized to initial protein level at the time of cycloheximide administration. Results shown are percent of protein remaining at each time point compared to control.

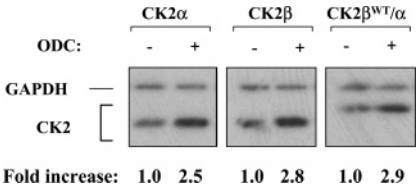


FIGURE 9: Effect of ODC overexpression on steady-state levels of CK2 mRNA. pIND-CK2β^{WT}/α clones were transiently transfected with either a pCX-derived control vector or pCXODC and induced with 10 μM ponasterone for 24 h. At this time, total RNA was isolated from these cells, and cDNA was prepared from each sample for semiquantitative PCR analysis. PCR reactions containing uniform quantities of ^{32}P-end-labeled primers were performed to analyze relative levels of the following transcripts: endogenous CK2α, endogenous CK2β, and the exogenous single transcript which encoded both wild-type CK2β and CK2α cDNAs. Signal strength of each band was detected by phosphorimager analysis, and the signal strength of the band from ODC-overexpressing cells was normalized to that present in the control vector for each product. Radiolabeled primers for GAPDH were also included in each PCR reaction to verify that equal amounts of cDNA were used in each reaction.

mechanisms of CK2 activation by polyamines in vivo have only now been addressed. Utilizing an EGFP-CK2 substrate whose phosphorylation by CK2 can be analyzed in transfected cells, we examined the role of the polyamines and the polyamine binding region of the CK2β regulatory subunit in the polyamine-dependent activation of CK2. Our results indicate that although polyamines do increase steady-state levels of both CK2α and CK2β subunits in the cell, neither activating mutations in the polyamine binding region nor the presence of increased polyamines (due to a dramatic increase in ODC activity) is sufficient to confer activation of CK2 in vivo. Rather, the increase in protein may be the result of increased transcription, as the overexpression of ODC increased steady-state levels of both exogenous and endogenous CK2α and CK2β mRNAs.

Our initial analysis of epitope-tagged CK2 tetramers immunoprecipitated from normal and ODC-overexpressing cells showed that increased levels of intracellular polyamines do not lead to any modification of wild-type CK2 which confers suprabasal kinase activity. This is perhaps not surprising, given that very few studies have been able to demonstrate increased CK2 kinase activity as the result of phosphorylation of the enzyme (*47*, *53*). ODC overexpression did cause an increase in a second, higher molecular weight

band recognized by an antibody directed against the CK2α hemagglutinin (HA) epitope (Figure 5). Such a mobility shift has been documented when CK2a is phosphorylated by the $p34^{cdc2}$ kinase in mitotic cells and may be important for the modulation of CK2α toward topoisomerase IIα by the peptide prolyl isomerase 1 (Pin1) (*57*). CK2 kinase activity was increased in immunocomplexes from ODC-overexpressing cells compared to those from normal cells; however, this increase directly paralleled the levels of immunoprecipitated CK2 subunits present in each reaction. Thus, it is also unlikely that, under these conditions, polyamines were directly interacting with CK2 to confer increased activity since polyamine-activated CK2 would be expected to exhibit an increased activation that was disproportionate to CK2 protein levels.

To more thoroughly address the intracellular effects of polyamines on CK2 activation, we transiently expressed an EGFP-based CK2 substrate protein (EGFP-S) whose phosphorylation by CK2 in vivo could be monitored prior to cell lysis. Unlike many of the canonical CK2 substrates such as B23, topoisomerase IIα, and nucleolin (*58*−*60*), which are phosphorylated by multiple kinases, the EGFP-CK2 fusion protein contains only a canonical consensus site for CK2 phosphorylation. The EGFP-S protein can also serve as an indicator of both cytoplasmic and nuclear CK2 activity since both EGFP and EGFP-S localize to both compartments of the cell (ref *54* and personal observation). Most importantly, CK2-mediated phosphorylation of the EGFP-S protein in vitro was stimulated by increasing levels of polyamines. This is in agreement with Leroy and others who have demonstrated that larger protein substrates require a conformational shift in the CK2 holoenzyme which occurs upon polyamine simulation, but that access of smaller molecules, such as the canonical CK2 peptide, to the active site is not sterically hindered and therefore unaffected by polyamine levels (*31*).

The in vivo phosphorylation of the EGFP-S CK2 substrate from cells overexpressing wild-type or mutant CK2 tetramers indicates that activating mutations in the polyamine binding site of CK2*β* are insufficient to confer increased phosphorylation of this substrate in vivo.

Although phosphorylation of EGFP-S was increased in ODC-overexpressing cells regardless of the CK2*β* subunit expressed, this phosphorylation could be accounted for by similar increases in CK2 holoenzyme, as evidenced by a 1.2−1.5-fold increase in CK2*β* levels. This is in agreement with immune complex kinase assays showing similar increases in both CK2 activity and CK2 protein levels in ODC-overexpressing cells. It has been demonstrated by Marin et al. (*61*) that the EGFP-S CK2 consensus site is a weak substrate for casein kinase 1 (CK1). Phosphorylation by CK1 seems unlikely to completely account for the polyamine-dependent phosphorylation of EGFP-S, however, as CK1 displayed an in vitro efficiency of phosphorylation toward the peptide version of the CK2 substrate that was an order of magnitude less than that of CK2 (*61*). In vivo increases in EGFP-S phosphorylation were accompanied by proportional increases in levels of CK2 subunits. The increase in steady-state levels of CK2*β* protein, however, was not the result of increased half-life, since the overexpression of ODC did not increase steady-state levels of CK2*β* in cycloheximide-treated cells.

Although a number of in vitro studies suggest that polyamines may directly bind to CK2 to increase kinase activity, our results suggest that the in vivo effect of polyamines on CK2 activity is chiefly accounted for by an increase in steady-state levels of both CK2α and CK2*β* transcripts as well as CK2 protein. The polyamine-dependent expression of CK2 is not surprising, since polyamines are known to modulate expression of a number of genes through their effect on nucleic acid structure which can affect multiple aspects of both transcription and translation (*62*).

The apparent discrepancy between the many reports of polyamine-dependent CK2 activation in vitro and the lack of increased phosphorylation of EGFP-S which we observed in ODC-overexpressing cells may be due to several unique aspects of polyamine metabolism that are not accurately accounted for by in vitro kinase assays. Perhaps the greatest contributor to a lack of in vivo activation of CK2 is that the polyamine species which accumulated to the greatest degree in ODC-overexpressing cells was putrescine, accounting for 80−90% of the increase in total polyamine levels. Levels of spermine, the most potent polyamine activator of CK2, increased only 4-fold. The diamine putrescine has the weakest affinity for the polyamine binding site of CK2 and thus stimulates its casein kinase activity to the least extent. It is almost completely unable to diplace polybasic effectors which bind to the same site on CK2*β* and stimulate the "calmodulin kinase" activity of CK2 (*34*).

The large fluctuations in putrescine levels, with much lesser changes in spermidine and spermine levels, are typical results of perturbations in ODC activity (*38*, *63*, *64*) and are a reflection of the numerous regulatory mechanisms which exist within the cell to maintain appropriate polyamine homeostasis. Polyamine overaccumulation can be tempered by catabolic enzymes, including the tandem of N1-spermidine/spermine acetyltransferase (N1-SSAT) and polyamine oxidase (*65*, *66*), as well as by export mechanisms for both acetylated longer chain amines and putrescine (*67*). It should be noted that both ODC and the N1-SSAT proteins can be phosphorylated by CK2 (*68*, *69*); however, phosphorylation by CK2 does not appear to alter the enzymatic activities of either enzyme (*70*, *71*). Although phosphorylation of ODC has been associated with increased stability, mutation of serine 303, the identified CK2 phosphorylation site on ODC, did not alter ODC turnover in mammalian cells (*70*). We expect that the phosphorylation is unlikely to influence levels of ODC in our studies, since we utilized an ODC which has already been stabilized by deletion of the PEST domain (*48*), and the stability of ODC that is offered by phosphorylation has been shown to be minimal in comparison (*48*, *72*).

A second factor unique to polyamine metabolism that may contribute to the lack of direct activation by CK2 in vivo is the distinct compartmentalization of polyamines within the cell. Studies of spermine distribution in liver suggest that as much as 90% of total intracellular polyamines may exist bound to RNA and the rest bound to DNA, ATP, and phospholipids (*51*). Less than 1% of total intracellular spermine was estimated to exist as a free species. This is in accordance with more recent immunoelectron microscopy studies demonstrating that polyamines in neurons are chiefly localized at both free and attached ribosomes of the rough endoplasmic reticulum (*73*). By this method, polyamines were found to be abundant in the cytoplasm of both neuronal

and neoplastic cells but were not detected in the nucleus (*73*, *74*). In contrast, CK2 α and β subunits are primarily localized to the nucleus (*75*, *76*). CK2 subunits have also been found to exist in both fast- and slow-moving pools, potentially reflecting the association of a subpopulation of CK2 with chromatin or nuclear matrix proteins (*54*). Nonnuclear CK2 may also be localized to specific sites, as CK2 has been found in association with membranes, cytoskeletal proteins, mitochondria, and extracellular substrates (*53*). Local polyamine concentrations within each compartment of the cell may not be sufficient to confer increased CK2 activation in vivo.

Polyamine compartmentalization may not be entirely responsible for this lack of activation, however, since overexpression of CK2 tetramers containing the CK2β mutant, engineered to mimic constituitively bound polyamines, was also unable to direct increased phosphorylation of the EGFP-S substrate compared to wild-type CK2. This may be accounted for by a limiting amount of holoenzyme formation. Several recent studies have led to the proposal that the formation of CK2 holoenzyme complexes is much more dynamic than previously thought (*53*, *54*). Free populations of CK2β have been found within cells (*75*, *77*), and CK2β appears to have several biologically relevant functions that do not rely on its association with CK2α (*29*, *78*, *79*). There is also evidence that a subpopulation of CK2α may also exist as a free form outside the tetrameric complex. Each subunit has been shown to independently translocate to the nucleus, a process that is downregulated upon formation of the holoenzyme tetramer (*54*). The polyamine-dependent stimulation of CK2 kinase activity is directly dependent on the formation of a holoenzyme tetramer and would have little effect on free CK2α subunits. In such a scenario, neither mutation of the polyamine binding site nor increases in polyamine levels would confer increased CK2 activity in vivo. This would also account for the fact that EGFP-S phosphorylation did reflect total CK2 protein levels in the cell but did not reflect the increased in vitro basal activity of the constituitively active CK2 mutant tetramer.

This work represents the first in vivo examination of the activation of CK2 by polyamines using a novel specific indicator of intracellular CK2 kinase activity. Although CK2 does not appear to be directly affected by polyamine levels in vivo, a large body of literature suggests that the activities of CK2 in the intracellular environment are no doubt influenced by such factors as subunit localization and trafficking, dynamics of holoenzyme assembly, ionic strength, and other protein−protein interactions. Despite the progress that has been made in elucidating the various functions of CK2, the mechanisms which underlie its own regulation within the cell are yet to be clearly defined. The EGFP-S substrate will allow a more accurate evaluation of both natural and synthetic modulators of CK2 activity in vivo, without the introduction of cell lysate-based artifacts, and should be particularly useful in this effort.

ACKNOWLEDGMENT

We thank Dr. Leonard Shore for the construction of the CK2β mutant cDNA and Dr. Thomas O'Brien for the critical review of the manuscript and helpful discussions. We gratefully acknowledge the assistance of Loretta Rossino in the preparation of the manuscript.

REFERENCES

1. Pinna, L. A. (1990) Casein kinase 2: an "eminence grise" in cellular regulation?, *Biochim. Biophys. Acta 1054*, 267−284.
2. Canton, D. A., Zhang, C., and Litchfield, D. W. (2001) Assembly of protein kinase CK2: investigation of complex formation between catalytic and regulatory subunits using a zinc-finger-deficient mutant of CK2beta, *Biochem. J. 358*, 87−94.
3. Filhol, O., Cochet, C., Loue-Mackenbach, P., and Chambaz, E. M. (1994) Oligomeric casein kinase II isoforms are expressed in bovine tissues and adrenocortical cells in culture, *Biochem. Biophys. Res. Commun. 198*, 660−665.
4. Cochet, C., and Chambaz, E. M. (1983) Oligomeric structure and catalytic activity of G type casein kinase. Isolation of the two subunits and renaturation experiments, *J. Biol. Chem. 258*, 1403−1406.
5. Meggio, F., and Pinna, L. A. (2003) One-thousand-and-one substrates of protein kinase CK2?, *FASEB J. 17*, 349−368.
6. Padmanabha, R., Chen-Wu, J. L., Hanna, D. E., and Glover, C. V. (1990) Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*, *Mol. Cell. Biol. 10*, 4089−4099.
7. Lorenz, P., Pepperkok, R., and Pyerin, W. (1994) Requirement of casein kinase 2 for entry into and progression through early phases of the cell cycle, *Cell Mol. Biol. Res. 40*, 519−527.
8. Lorenz, P., Pepperkok, R., Ansorge, W., and Pyerin, W. (1993) Cell biological studies with monoclonal and polyclonal antibodies against human casein kinase II subunit beta demonstrate participation of the kinase in mitogenic signaling, *J. Biol. Chem. 268*, 2733−2739.
9. Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D. C., Traish, A. M., Mercurio, F., and Sonenshein, G. E. (2001) Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-kappaB in breast cancer, *Cancer Res. 61*, 3810−3818.
10. Yenice, S., Davis, A. T., Goueli, S. A., Akdas, A., Limas, C., and Ahmed, K. (1994) Nuclear casein kinase 2 (CK-2) activity in human normal, benign hyperplastic, and cancerous prostate, *Prostate 24*, 11−16.
11. Munstermann, U., Fritz, G., Seitz, G., Lu, Y. P., Schneider, H. R., and Issinger, O. G. (1990) Casein kinase II is elevated in solid human tumours and rapidly proliferating non-neoplastic tissue, *Eur. J. Biochem. 189*, 251−257.
12. Faust, R. A., Gapany, M., Tristani, P., Davis, A., Adams, G. L., and Ahmed, K. (1996) Elevated protein kinase CK2 activity in chromatin of head and neck tumors: association with malignant transformation, *Cancer Lett. 101*, 31−35.
13. Bousset, K., Oelgeschlager, M. H., Henriksson, M., Schreek, S., Burkhardt, H., Litchfield, D. W., Luscher-Firzlaff, J. M., and Luscher, B. (1994) Regulation of transcription factors c-Myc, Max, and c-Myb by casein kinase II, *Cell Mol. Biol. Res. 40*, 501−511.
14. Filhol, O., Baudier, J., Delphin, C., Loue-Mackenbach, P., Chambaz, E. M., and Cochet, C. (1992) Casein kinase II and the tumor suppressor protein P53 associate in a molecular complex that is negatively regulated upon P53 phosphorylation, *J. Biol. Chem. 267*, 20577−20583.
15. O'Brien, K. A., Lemke, S. J., Cocke, K. S., Rao, R. N., and Beckmann, R. P. (1999) Casein kinase 2 binds to and phosphorylates BRCA1, *Biochem. Biophys. Res. Commun. 260*, 658−664.
16. Landesman-Bollag, E., Channavajhala, P. L., Cardiff, R. D., and Seldin, D. C. (1998) p53 deficiency and misexpression of protein kinase CK2alpha collaborate in the development of thymic lymphomas in mice, *Oncogene 16*, 2965−2974.
17. Krippner-Heidenreich, A., Talanian, R. V., Sekul, R., Kraft, R., Thole, H., Ottleben, H., and Luscher, B. (2001) Targeting of the transcription factor Max during apoptosis: phosphorylation-regulated cleavage by caspase-5 at an unusual glutamic acid residue in position P1, *Biochem. J. 358*, 705−715.
18. Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E., Vilbois, F., Hochmann, A., Journot, L., Antonsson, B., and Martinou, J. C. (2001) Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8, *Mol. Cell 8*, 601−611.
19. Yin, X., Gu, S., and Jiang, J. X. (2001) Regulation of lens connexin 45.6 by apoptotic protease, caspase-3, *Cell Commun. Adhes. 8*, 373−376.
20. Ruzzene, M., Penzo, D., and Pinna, L. A. (2002) Protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induces

apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells, *Biochem. J. 364*, 41−47.
21. Wang, H., Davis, A., Yu, S., and Ahmed, K. (2001) Response of cancer cells to molecular interruption of the CK2 signal, *Mol. Cell. Biochem. 227*, 167−174.
22. Guo, C., Yu, S., Davis, A. T., Wang, H., Green, J. E., and Ahmed, K. (2001) A potential role of nuclear matrix-associated protein kinase CK2 in protection against drug-induced apoptosis in cancer cells, *J. Biol. Chem. 276*, 5992−5999.
23. Litchfield, D. W. (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death, *Biochem. J. 369*, 1−15.
24. Farah, M., Parhar, K., Moussavi, M., Eivemark, S., and Salh, B. (2003) 5,6-Dichloro-ribifuranosylbenzimidazole- and apigenin-induced sensitization of colon cancer cells to TNF-alpha-mediated apoptosis, *Am. J. Physiol. Gastrointest. Liver Physiol. 285*, G919−G928.
25. Slaton, J. W., Unger, G. M., Sloper, D. T., Davis, A. T., and Ahmed, K. (2004) Induction of apoptosis by antisense CK2 in human prostate cancer xenograft model, *Mol. Cancer Res. 2*, 712−721.
26. Pegg, A. E., and McCann, P. P. (1982) Polyamine metabolism and function, *Am. J. Physiol. 243*, C212−C221.
27. Tabor, C. W., and Tabor, H. (1984) Polyamines, *Annu. Rev. Biochem. 53*, 749−790.
28. Issinger, O. G. (1993) Casein kinases: pleiotropic mediators of cellular regulation, *Pharmacol. Ther. 59*, 1−30.
29. Allende, J. E., and Allende, C. C. (1995) Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation, *FASEB J. 9*, 313−323.
30. Leroy, D., Schmid, N., Behr, J. P., Filhol, O., Pares, S., Garin, J., Bourgarit, J. J., Chambaz, E. M., and Cochet, C. (1995) Direct identification of a polyamine binding domain on the regulatory subunit of the protein kinase casein kinase 2 by photoaffinity labeling, *J. Biol. Chem. 270*, 17400−17406.
31. Leroy, D., Heriche, J. K., Filhol, O., Chambaz, E. M., and Cochet, C. (1997) Binding of polyamines to an autonomous domain of the regulatory subunit of protein kinase CK2 induces a conformational change in the holoenzyme. A proposed role for the kinase stimulation, *J. Biol. Chem. 272*, 20820−20827.
32. Meggio, F., Boldyreff, B., Issinger, O. G., and Pinna, L. A. (1994) Casein kinase 2 down-regulation and activation by polybasic peptides are mediated by acidic residues in the 55−64 region of the beta-subunit. A study with calmodulin as phosphorylatable substrate, *Biochemistry 33*, 4336−4342.
33. Niefind, K., Guerra, B., Ermakowa, I., and Issinger, O. G. (2001) Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme, *EMBO J. 20*, 5320−5331.
34. Sarno, S., Marin, O., Meggio, F., and Pinna, L. A. (1993) Polyamines as negative regulators of casein kinase-2: the phosphorylation of calmodulin triggered by polylysine and by the alpha[66−86] peptide is prevented by spermine, *Biochem. Biophys. Res. Commun. 194*, 83−90.
35. Boldyreff, B., Meggio, F., Pinna, L. A., and Issinger, O. G. (1993) Reconstitution of normal and hyperactivated forms of casein kinase-2 by variably mutated beta-subunits, *Biochemistry 32*, 12672−12677.
36. Hathaway, G. M., and Traugh, J. A. (1984) Interaction of polyamines and magnesium with casein kinase II, *Arch Biochem. Biophys. 233*, 133−138.
37. Meggio, F., Grankowski, N., Kudlicki, W., Szyszka, R., Gasior, E., and Pinna, L. A. (1986) Structure and properties of casein kinase-2 from *Saccharomyces cerevisiae*. A comparison with the liver enzyme, *Eur. J. Biochem. 159*, 31−38.
38. Shore, L. J., Soler, A. P., and Gilmour, S. K. (1997) Ornithine decarboxylase expression leads to translocation and activation of protein kinase CK2 in vivo, *J. Biol. Chem. 272*, 12536−12543.
39. Praskova, M., Kalenderova, S., Miteva, L., Poumay, Y., and Mitev, V. (2002) The ornithine decarboxylase inhibitor, difluoromethylornithine, inhibits casein kinase II activity, c-Myc expression and normal human keratinocyte proliferation, *Arch. Dermatol. Res. 293*, 590−593.
40. Siemer, S., Kriener, S., Konig, J., Remberger, K., and Issinger, O. G. (1995) Influence of indomethacin and difluoromethylornithine on human tumour growth in nude mice, *Eur. J. Cancer 31A*, 976−981.
41. Clifford, A., Morgan, D., Yuspa, S. H., Soler, A. P., and Gilmour, S. (1995) Role of ornithine decarboxylase in epidermal tumorigenesis, *Cancer Res. 55*, 1680−1686.
42. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997) "Green mice" as a source of ubiquitous green cells, *FEBS Lett. 407*, 313−319.
43. Megosh, L., Gilmour, S. K., Rosson, D., Soler, A. P., Blessing, M., Sawicki, J. A., and O'Brien, T. G. (1995) Increased frequency of spontaneous skin tumors in transgenic mice which overexpress ornithine decarboxylase, *Cancer Res. 55*, 4205−4209.
44. Bek, S., and Kemler, R. (2002) Protein kinase CKII regulates the interaction of beta-catenin with alpha-catenin and its protein stability, *J. Cell Sci. 115*, 4743−4753.
45. Gietz, R. D., Graham, K. C., and Litchfield, D. W. (1995) Interactions between the subunits of casein kinase II, *J. Biol. Chem. 270*, 13017−13021.
46. Sayed, M., Kim, S. O., Salh, B. S., Issinger, O. G., and Pelech, S. L. (2000) Stress-induced activation of protein kinase CK2 by direct interaction with p38 mitogen-activated protein kinase, *J. Biol. Chem. 275*, 16569−16573.
47. Donella-Deana, A., Cesaro, L., Sarno, S., Ruzzene, M., Brunati, A. M., Marin, O., Vilk, G., Doherty-Kirby, A., Lajoie, G., Litchfield, D. W., and Pinna, L. A. (2003) Tyrosine phosphorylation of protein kinase CK2 by Src-related tyrosine kinases correlates with increased catalytic activity, *Biochem. J. 372*, 841−849.
48. Ghoda, L., van Daalen Wetters, T., Macrae, M., Ascherman, D., and Coffino, P. (1989) Prevention of rapid intracellular degradation of ODC by a carboxyl-terminal truncation, *Science 243*, 1493−1495.
49. Leroy, D., Filhol, O., Delcros, J. G., Pares, S., Chambaz, E. M., and Cochet, C. (1997) Chemical features of the protein kinase CK2 polyamine binding site, *Biochemistry 36*, 1242−1250.
50. Paulus, T. J., Cramer, C. L., and Davis, R. H. (1983) Compartmentation of spermidine in *Neurospora crassa*, *J. Biol. Chem. 258*, 8608−8612.
51. Watanabe, S., Kusama-Eguchi, K., Kobayashi, H., and Igarashi, K. (1991) Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver, *J. Biol. Chem. 266*, 20803−20809.
52. Tabor, C. W., and Kellogg, P. D. (1967) The effect of isolation conditions on the polyamine content of *Escherichia coli* ribosomes, *J. Biol. Chem. 242*, 1044−1052.
53. Olsten, M. E., and Litchfield, D. W. (2004) Order or chaos? An evaluation of the regulation of protein kinase CK2, *Biochem. Cell Biol. 82*, 681−693.
54. Filhol, O., Nueda, A., Martel, V., Gerber-Scokaert, D., Benitez, M. J., Souchier, C., Saoudi, Y., and Cochet, C. (2003) Live-cell fluorescence imaging reveals the dynamics of protein kinase CK2 individual subunits, *Mol. Cell. Biol. 23*, 975−987.
55. Yang, F., Liu, Y., Bixby, S. D., Friedman, J. D., and Shokat, K. M. (1999) Highly efficient green fluorescent protein-based kinase substrates, *Anal. Biochem. 266*, 167−173.
56. Zhang, C., Vilk, G., Canton, D. A., and Litchfield, D. W. (2002) Phosphorylation regulates the stability of the regulatory CK2beta subunit, *Oncogene 21*, 3754−3764.
57. Messenger, M. M., Saulnier, R. B., Gilchrist, A. D., Diamond, P., Gorbsky, G. J., and Litchfield, D. W. (2002) Interactions between protein kinase CK2 and Pin1. Evidence for phosphorylation-dependent interactions, *J. Biol. Chem. 277*, 23054−23064.
58. Isaacs, R. J., Davies, S. L., Sandri, M. I., Redwood, C., Wells, N. J., and Hickson, I. D. (1998) Physiological regulation of eukaryotic topoisomerase II, *Biochim. Biophys. Acta 1400*, 121−137.
59. Jiang, P. S., Chang, J. H., and Yung, B. Y. (2000) Different kinases phosphorylate nucleophosmin/B23 at different sites during G(2) and M phases of the cell cycle, *Cancer Lett. 153*, 151−160.
60. Belenguer, P., Caizergues-Ferrer, M., Labbe, J. C., Doree, M., and Amalric, F. (1990) Mitosis-specific phosphorylation of nucleolin by p34cdc2 protein kinase, *Mol. Cell. Biol. 10*, 3607−3618.
61. Marin, O., Meggio, F., and Pinna, L. A. (1994) Design and synthesis of two new peptide substrates for the specific and sensitive monitoring of casein kinases-1 and -2, *Biochem. Biophys. Res. Commun. 198*, 898−905.
62. Igarashi, K., and Kashiwagi, K. (2000) Polyamines: mysterious modulators of cellular functions, *Biochem. Biophys. Res. Commun. 271*, 559−564.
63. Qu, N., Ignatenko, N. A., Yamauchi, P., Stringer, D. E., Levenson, C., Shannon, P., Perrin, S., and Gerner, E. W. (2003) Inhibition

of human ornithine decarboxylase activity by enantiomers of difluoromethylornithine, *Biochem. J. 375*, 465−470.
64. Smith, M. K., Trempus, C. S., and Gilmour, S. K. (1998) Cooperation between follicular ornithine decarboxylase and v-Ha-ras induces spontaneous papillomas and malignant conversion in transgenic skin, *Carcinogenesis 19*, 1409−1415.
65. Casero, R. A., Jr., and Pegg, A. E. (1993) Spermidine/spermine N1-acetyltransferase—the turning point in polyamine metabolism, *FASEB J. 7*, 653−661.
66. Seiler, N. (2004) Catabolism of polyamines, *Amino Acids 26*, 217−233.
67. Xie, X., Gillies, R. J., and Gerner, E. W. (1997) Characterization of a diamine exporter in Chinese hamster ovary cells and identification of specific polyamine substrates, *J. Biol. Chem. 272*, 20484−20489.
68. Meggio, F., Flamigni, F., Caldarera, C. M., Guarnieri, C., and Pinna, L. A. (1984) Phosphorylation of rat heart ornithine decarboxylase by type-2 casein kinase, *Biochem. Biophys. Res. Commun. 122*, 997−1004.
69. Bordin, L., Vargiu, C., Colombatto, S., Clari, G., Testore, G., Toninello, A., and Grillo, M. A. (1996) Casein kinase 2 phosphorylates recombinant human spermidine/spermine N1-acetyltransferase on both serine and threonine residues, *Biochem. Biophys. Res. Commun. 229*, 845−851.
70. Rosenberg-Hasson, Y., Strumpf, D., and Kahana, C. (1991) Mouse ornithine decarboxylase is phosphorylated by casein kinase-II at a predominant single location (serine 303), *Eur. J. Biochem. 197*, 419−424.
71. Bordin, L., Vargiu, C., Clari, G., Brunati, A. M., Colombatto, S., Salvi, M., Grillo, M. A., and Toninello, A. (2002) Phosphorylation of recombinant human spermidine/spermine N(1)-acetyltransferase by CK1 and modulation of its binding to mitochondria: a comparison with CK2, *Biochem. Biophys. Res. Commun. 290*, 463−468.
72. Reddy, S. G., McLlheran, S. M., Cochran, B. J., Worth, L. L., Bishop, L. A., Brown, P. J., Knutson, V. P., and Haddox, M. K. (1996) Multisite phosphorylation of ornithine decarboxylase in transformed macrophages results in increased intracellular enzyme stability and catalytic efficiency, *J. Biol. Chem. 271*, 24945−24953.
73. Fujiwara, K., Bai, G., Kitagawa, T., and Tsuru, D. (1998) Immunoelectron microscopic study for polyamines, *J. Histochem. Cytochem. 46*, 1321−1328.
74. Fujiwara, K., Furukawa, K., Nakayama, E., and Shiku, H. (1994) Production and characterization of monoclonal antibodies against the polyamine, spermine: immunocytochemical localization in rat tissues, *Histochemistry 102*, 397−404.
75. Krek, W., Maridor, G., and Nigg, E. A. (1992) Casein kinase II is a predominantly nuclear enzyme, *J. Cell Biol. 116*, 43−55.
76. Martel, V., Filhol, O., Nueda, A., Gerber, D., Benitez, M. J., and Cochet, C. (2001) Visualization and molecular analysis of nuclear import of protein kinase CK2 subunits in living cells, *Mol. Cell. Biochem. 227*, 81−90.
77. Guerra, B., Siemer, S., Boldyreff, B., and Issinger, O. G. (1999) Protein kinase CK2: evidence for a protein kinase CK2beta subunit fraction, devoid of the catalytic CK2alpha subunit, in mouse brain and testicles, *FEBS Lett. 462*, 353−357.
78. Chen, M., Li, D., Krebs, E. G., and Cooper, J. A. (1997). The casein kinase II beta subunit binds to Mos and inhibits Mos activity, *Mol. Cell. Biol. 17*, 1904−1912.
79. Hagemann, C., Kalmes, A., Wixler, V., Wixler, L., Schuster, T., and Rapp, U. R. (1997) The regulatory subunit of protein kinase CK2 is a specific A-Raf activator, *FEBS Lett. 403*, 200−202.

BI052480I