

Protein kinase CK2 as an ectokinase: The role of the regulatory CK2 β subunit

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Protein kinase CK2 (also known as casein kinase 2) is present in the cytoplasm, nuclei, and several other organelles. In addition, this enzyme has been found bound to the external side of the cell membrane where it acts as an ectokinase phosphorylating several extracellular proteins. Previous experiments with transfection of HEK-293T cells demonstrated that expression of both subunits, CK2 α (catalytic) and CK2 β (regulatory), was necessary for the appearance of the ectopic enzyme as an ectokinase. In this work, using deletion and point mutations of CK2 β , it was possible to demonstrate that the region between amino acids 20 and 33 was necessary for the export of the enzyme as an ectokinase. Phenylalanines 21 and 22 and acidic residues in positions 26–28 are involved in the structural aspects that are required for export. However, the region encompassing amino acids 20–33 of CK2 β is not sufficient to make the carboxyl half of this subunit functional in bringing CK2 to the ectokinase locus. In cells transfected with only CK2 β , it was demonstrated that 3–4% of the subunit is exported to the cell medium, but the subunit is not bound to the external membrane.

casein kinase 2 | export of proteins | shedding

It has been known for a number of years that protein kinase CK2 (formerly called casein kinase 2) is present in the cytoplasm, nuclei, and several other cell organelles. It has also been found on the external side of the cellular membrane where, acting as an ectokinase, it can phosphorylate extracellular proteins and external domains of proteins. Kubler *et al.* (1) originally discovered that incubation of cells with CK2 substrates such as casein or phosphatidylcholine resulted in the liberation of the enzyme activity into the medium. This substrate-mediated release from the cell membrane was called “shedding.”

Several extracellular proteins have been identified as substrates of the CK2 ectokinase activity. Vitronectin has been shown to be phosphorylated extracellularly by CK2, and evidence has been presented to demonstrate that its phosphorylation regulates the adhesion of cells to the extracellular matrix (2, 3). More recently, it has been shown that CK2 phosphorylates the C9 complement and it has been suggested that this phosphorylation might control cell lysis caused by this complement protein (4). Another recent example of an extracellular domain phosphorylated by ecto CK2 is the collagen XVII receptor. This phosphorylation may inhibit its degradation catalyzed by metalloproteases (5).

In our previous work (6), we explored the conditions and requirements necessary to observe the presence of ectopically expressed CK2 holoenzyme that could be obtained after shedding of human cells in culture that had been transfected with the cDNAs coding for the two CK2 subunits. This work demonstrated that transfection with both catalytic (CK2 α) and regulatory (CK2 β) subunits was necessary to detect ectopically expressed CK2 bound externally to the cellular membrane. It was further shown that the export outside the cell did not require catalytically active enzyme and that the appearance as an ectokinase was 5–7 h delayed with respect to its synthesis inside the cell.

In the present work, the role of the CK2 β subunit in the export of the holoenzyme to the extracellular membrane is explored through the use of deletion and point mutations. The region of CK2 β between amino acids 20 and 33 was found to be necessary but not sufficient to allow the catalytic subunit to function as an ectokinase. Experiments are also presented that indicate that free CK2 β can be exported out of the cell but is not retained bound to the cell membrane. These findings raise the possibility that CK2 β may act as a carrier for the export of proteins that are able to bind tightly.

Results and Discussion

Regions of CK2 β Involved in CK2 Ectokinase Location. Fig. 1 shows the series of mutations that have been prepared to generate modified CK2 β . All of these constructs result in proteins that contain six Myc epitope tags in tandem. The mutations were designed to maintain intact the carboxyl half of CK2 β because this portion has been shown to be essential for its dimerization and binding to CK2 α and the formation of the holoenzyme (7, 8).

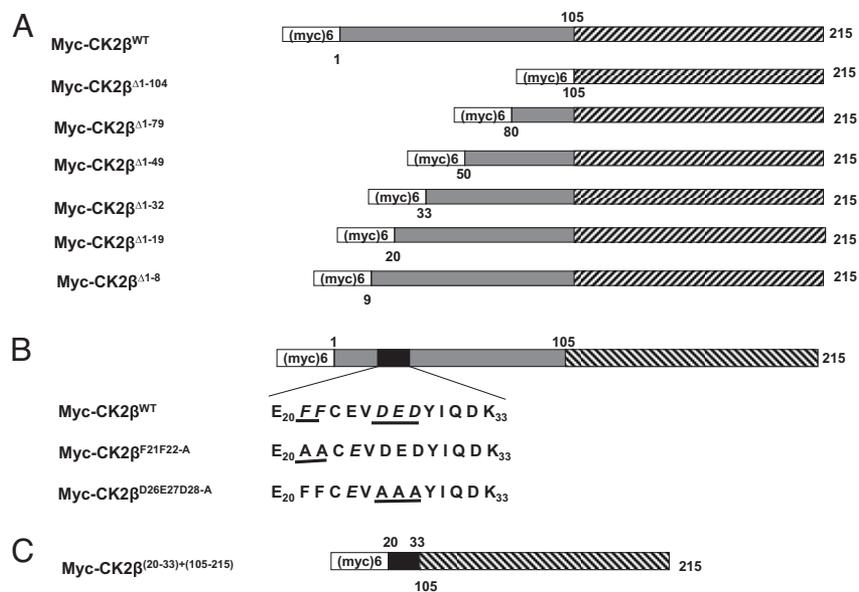
These constructs in vector pCS2+MT were transfected into HEK293T cells together with HA-CK2 α in pCEFL-HA vectors that yield HA epitope-tagged ectopic proteins. Twenty-four hours after transfections the cells were exposed to the shedding conditions and subsequently lysed. As described in *Materials and Methods*, shedding liquid and cell lysates were immunoprecipitated with anti-HA antibodies, and the catalytic activity present in the immunoprecipitates was assayed by using a CK2-specific peptide substrate.

Fig. 2 shows the results obtained with three deletion mutations of CK2 β and controls carried out with the wild-type (CK2 β) subunit and with HA-CK2 α alone. Fig. 2A shows that immunoprecipitates of cell lysates obtained with two different constructs have the same level of CK2 activity as those transfected with wild-type CK2 β , except that transfection with HA-CK2 α alone gives significantly lower activity.

Fig. 2B shows ectokinase activity of the same cells whose lysates are analyzed in Fig. 2A, and it can be seen that the (Myc)₆-CK2 β ^{Δ 1–8} construct that lacks the first eight amino acids of the regulatory subunit was able to generate as much CK2 ectokinase activity as the wild-type subunit. Cells transfected with mutant (Myc)₆-CK2 β ^{Δ 1–32}, on the other hand, did not have significant ectokinase activity, just as cells that were transfected only with Ha-CK2 α ^{WT}. Fig. 2C and D shows a similar, but separate, experiment run with the (Myc)₆-CK2 β ^{Δ 1–19}. Fig. 2C shows the ectopic CK2 activity of cell lysates transfected with wild-type and mutant CK2 β ^{Δ 1–19}, and Fig. 2D shows the CK2 ectokinase activity obtained through shedding of these same transfected cells. Fig. 2D shows that transfection with (Myc)₆-CK2 β ^{Δ 1–19} yields as much ectokinase activity as observed with

Author contributions: J.E.A. designed research; F.A.R. and C.C. performed research; V.B.-G. contributed new reagents/analytic tools; V.B.-G. and J.E.A. analyzed data; and J.E.A. wrote the paper.

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the wild-type subunit. The possibility that the (Myc)₆ tag was affecting the results was discarded by experiments in which the (Myc)₆ tag was replaced for an HA tag attached to CK2 β constructs with deletion mutants that yielded ectokinase activity and no ectokinase activity. The results observed with the HA tags were similar to those found with the (Myc)₆ tags (data not shown).

Lysate extracts from the cells transfected with the wild-type and mutant constructs of (Myc)₆ CK2 β and the corresponding anti-HA immunoprecipitates were electrophoresed and analyzed by Western blots by using anti-Myc antibodies (Fig. 2E). The direct anti-Myc Western blot analysis of the extracts (Fig. 2E, lanes 1, 3, 5, 7, and 9) allowed us to determine that the different CK2 β mutants had been expressed in these cells in amounts similar to the wild-type protein. This observation means that the absence of ectokinase activity in cells transfected with CK2 $\beta^{\Delta 1-32}$ was not caused by their lack of expression or instability. In Fig. 2E, lanes 2, 4, 6, 8, and 10 it is possible to see that the antibodies directed against the HA tag present in HA-CK2 α were able to coimmunoprecipitate the (Myc)₆-tagged CK2 β constructs as revealed by the anti-Myc antibodies. These results mean that these mutant CK2 β proteins coimmunoprecipitate with the catalytic subunit and therefore can form strong associations with CK2 α and probably attain a holoenzyme structure (9). Previous work in the laboratories of Pinna and Issinger (10, 11) with CK2 β fragments also demonstrated that amino-truncated CK2 β could interact with and stimulate CK2 α . In separate experiments, cells were transfected with (Myc)₆-CK2 $\beta^{\Delta 1-49}$, (Myc)₆-CK2 $\beta^{\Delta 1-79}$, and (Myc)₆-CK2 $\beta^{\Delta 1-104}$ together with HA-CK2 α . These three other deletion mutants also failed to generate detectable CK2 ectokinase activity (data not shown).

The results described in Fig. 2 indicate that the CK2 β region between amino acids 20 and 33 is important for this subunit to cause the appearance of CK2 as an ectokinase. In this 14-aa stretch (Fig. 1B), one can notice two salient features: a pair of contiguous phenylalanine residues (F²¹, F²²) and an acidic cluster (E²⁴, D²⁶, E²⁷, D²⁸). To test the participation of these amino acids, point mutations were prepared in full-length

(Myc)₆-CK2 β constructs. As seen in Fig. 1B, mutant Myc-CK2 $\beta^{\text{F21F22-A}}$ contains F²¹ and F²² mutated to alanine, and mutant Myc-CK2 $\beta^{\text{D26E27D28-A}}$ has D²⁶, E²⁷, and D²⁸ mutated to alanine. These mutants were transfected into cells (Fig. 3A and B), and the amount of activity was measured in the lysates and the ectokinase locus. The activity detected in the lysates (Fig. 3A) with the mutant CK2 β constructs was somewhat lower than in the lysates with the wild-type CK2 β subunit, especially in the case of CK2 $\beta^{\text{F21F22-A}}$, which was 30% reduced over the wild-type control. Even considering this fact, the activity found in the ectokinase locus was much more significantly affected as compared with the wild-type subunit (75% reduction in the case of CK2 $\beta^{\text{F21F22-A}}$). Fig. 3C shows that both mutants were expressed well in cell lysates and also coimmunoprecipitated with CK2 α . The reason for the marked effect in the mutant that affects phenylalanines 21 and 22 was investigated in a structural model. These two aromatic amino acids in the crystal structure are interacting with other residues. Fig. 4A shows the interactions of F²¹ that forms a hydrogen bond with H¹⁵¹ and hydrophobic interaction with F¹⁰⁶. In Fig. 4B, we see that F²² interacts through a hydrogen bond with N¹⁹ and hydrophobic interaction with F¹⁵⁹. These bonds indicate that these two phenylalanine residues play an important role in anchoring the loop, which contains the 20–33 sequence important for the export of the protein, to the rest of the molecule. On the other hand, the acidic residues (E²⁴, D²⁶, E²⁷, and D²⁸) are exposed to the medium and are free to interact with other proteins (12).

The results above demonstrated that the region of CK2 β between amino acids 20 and 33 is necessary for CK2 holoenzyme to appear as an ectokinase. To address the question of whether this region is also sufficient to endow a protein with the capacity to bring the catalytic subunit to the ectokinase locus, a construct was made in which the 20–33 peptide was covalently linked to the 105–215 carboxyl half of CK2 β (Fig. 1C). This construct was able to bind tightly to HA-CK2 α because it was coimmunoprecipitated with this subunit when the HA antibody was used for immunoprecipitation of the cell lysate (data not shown). However, as seen in Fig. 5, the product of this construct was not able

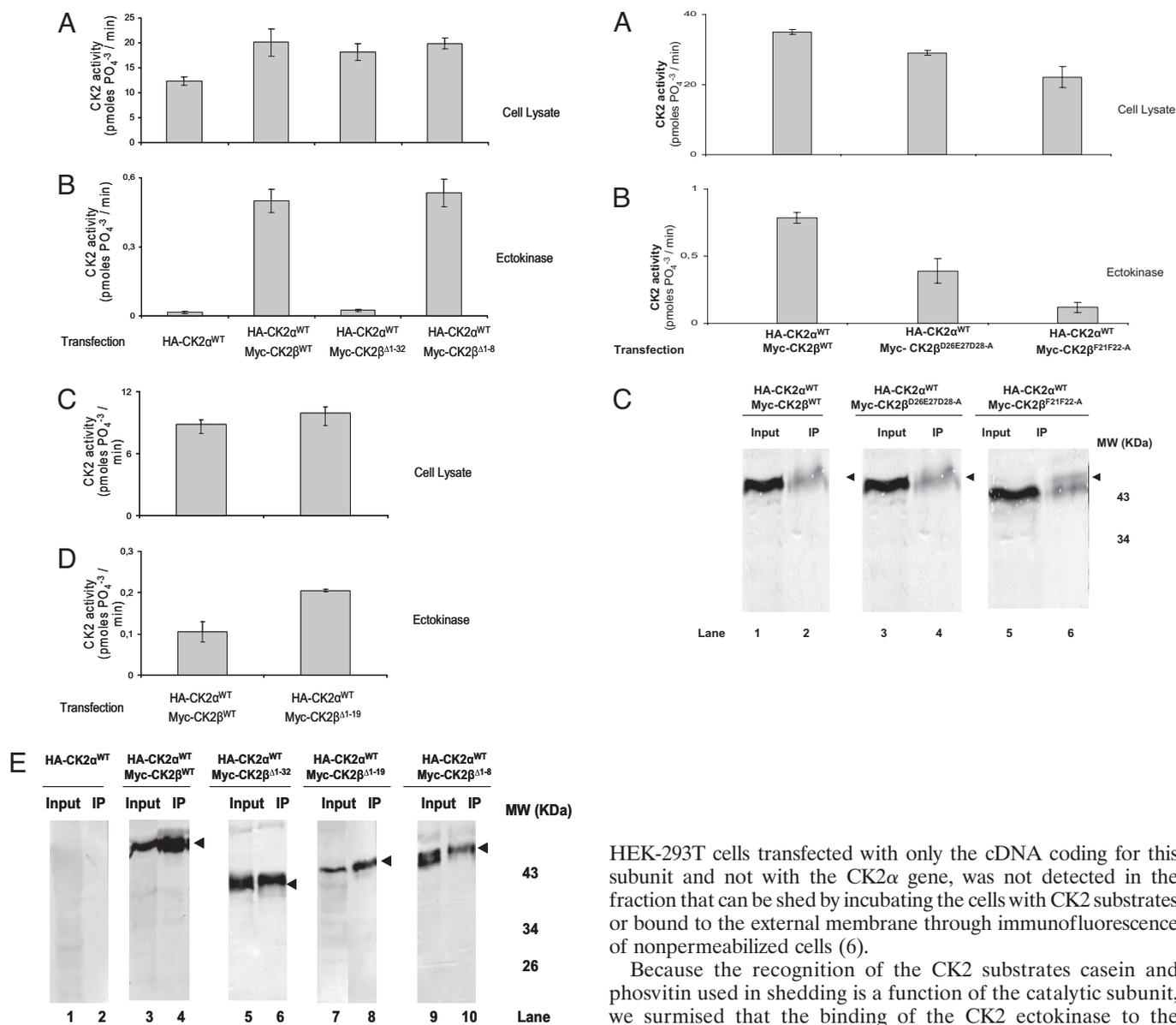
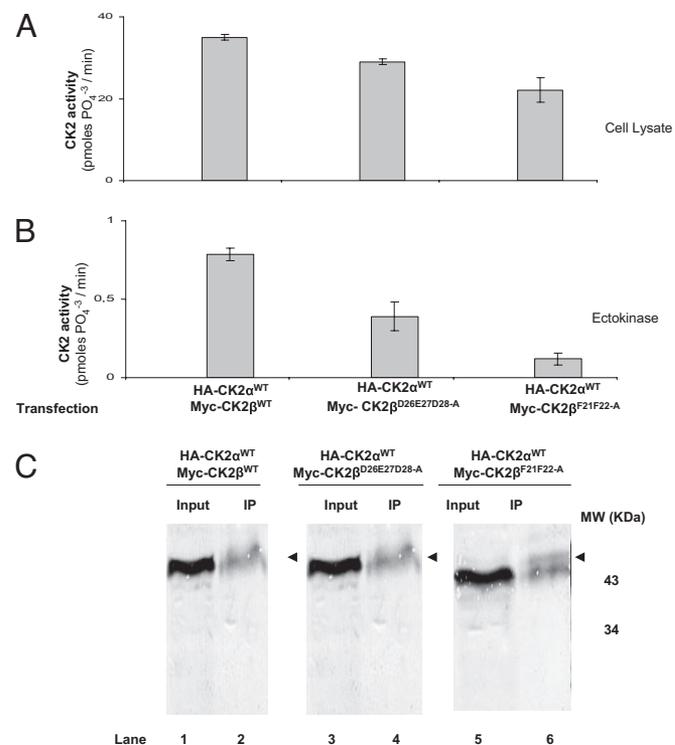


Fig. 2. CK2 activity and Western blots in cells transfected with CK2 α and CK2 β constructs. (A–D) CK2 activity measured in anti-HA immunoprecipitates of cell lysates (A and C) and the extracellular liquid obtained after the shedding procedure, which defines ectokinase (B and D). The constructs used for transfection are at the bottom of B and D. The activity scales, measured as described in *Materials and Methods*, indicate pmols of radioactive phosphoryl groups incorporated per minute into the specific CK2 peptide substrate. The bars show the variations obtained in separate experiments (2) carried out under similar conditions. (E) Shown are the Western blots obtained after lysates (input) or immunoprecipitates (IP) of cell lysates exposed to anti-HA antibodies are fractionated on SDS/PAGE and probed with anti-Myc antibodies as described in *Materials and Methods*. The constructs used to transfect the cells are indicated on top of each pair of Western blots.

to cause the appearance of CK2 activity in the ectokinase compartment and behaved similarly to the deletion mutant Myc-CK2 $\beta^{\Delta 1-104}$. The presence of the peptide between amino acids 20 and 33 is therefore not sufficient to elicit the presence of CK2 in the ectokinase location.

Free CK2 β Can Be Exported Out of the Cell but Is Not Bound to the Extracellular Membrane. In our previous work, we had presented evidence that free CK2 β , generated by expression of this subunit in



HEK-293T cells transfected with only the cDNA coding for this subunit and not with the CK2 α gene, was not detected in the fraction that can be shed by incubating the cells with CK2 substrates or bound to the external membrane through immunofluorescence of nonpermeabilized cells (6).

Because the recognition of the CK2 substrates casein and phosphotyrosine used in shedding is a function of the catalytic subunit, we surmised that the binding of the CK2 ectokinase to the extracellular membrane might be a function of the catalytic subunit. This idea led us to search for free Myc-CK2 β in the culture medium and in the wash of cells transfected with the cDNA coding for this subunit. Because CK2 β has no catalytic activity and only a small fraction may be exported outside cells, a sensitive method for its detection had to be devised. This method consisted of immunoprecipitation of the culture medium and the subsequent wash fractions using anti-Myc antibodies. The putative Myc-CK2 β in the immunoprecipitate was combined with bacterially synthesized recombinant CK2 α to reconstitute the holoenzyme, which was subsequently incubated with [γ -³²P]ATP to allow autophosphorylation of the CK2 β in serines 2 and 3 of this subunit (9, 13). A faint radioactive band was observed to coincide with the mobility of (Myc)₁-CK2 β in the culture medium (Fig. 6, lane 11). To ascertain that this band corresponded to the CK2 regulatory subunit, a construct was made that contained six Myc epitopes in tandem fused to CK2 β that had been used to tag all of the previous constructs of CK2 β . This larger (Myc)₆-CK2 β generates a protein with slower mobility in SDS/PAGE that is equivalent to a 45-kDa protein (Fig. 6, lane 10). When this (Myc)₆-CK2 β construct was used for cell transfection, the corresponding radioactive band was observed in the culture medium and first wash liquid of the cells immuno-

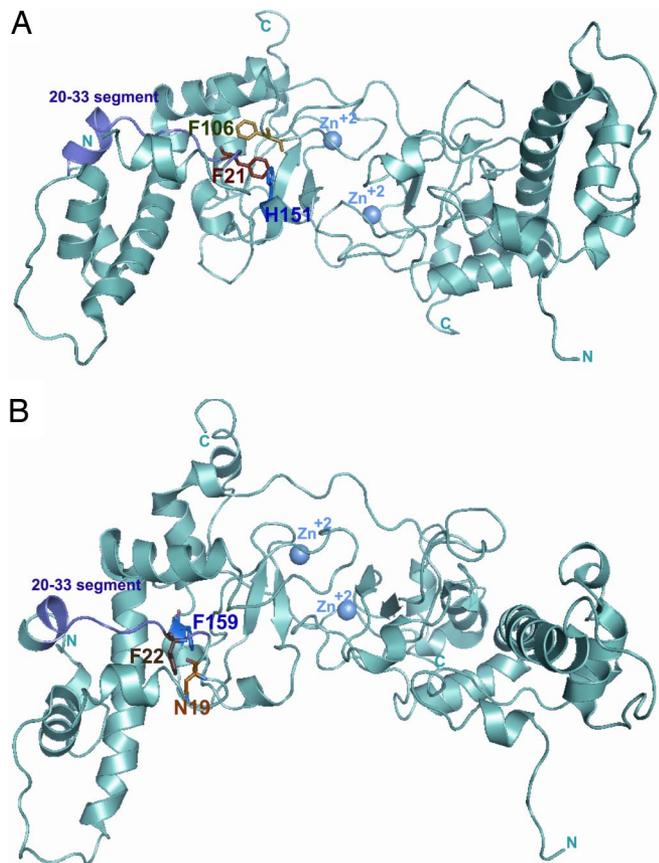


Fig. 4. Position and interactions of F21 and F22 in the CK2 β dimers. The 3D structure of the CK2 β dimer (20) is presented with segment 20–33 shown in purple for subunit on left. (A) The hydrogen bond contact of F21 (red) with H151 (dark blue) and hydrophobic interaction with F106 (orange) are shown. (B) The hydrogen bond of F22 (red) with N19 (orange) and hydrophobic interaction with F159 (dark blue) are shown.

precipitated and autophosphorylated (Fig. 6, lanes 2 and 4), but was not found in the second wash or in the liquid obtained after the shedding extraction of the cells (Fig. 6, lanes 6 and 8). The ectopic regulatory subunit was apparently free of catalytic subunits because no autophosphorylation could be detected when exogenous catalytic subunits were not added to the immunoprecipitates (Fig. 6, lanes 1, 3, 5, and 7). The intensity of the radioactive bands as compared with a standard curve with a range of concentrations of (Myc) $_6$ CK2 β allowed us to calculate that \approx 3–4% of the (Myc) $_6$ CK2 β present in the lysate of these cells can be found in the extracellular medium and in the first wash. This percentage is comparable to the proportion of CK2 found as an ectokinase when cells are cotransfected with the cDNAs of both subunits. Thus, it appears that CK2 β by itself has the property to be exported out of cells. After transfections of cells with HA-CK2 α alone, attempts to detect catalytic subunits in the cell medium or in the wash liquids through immunoprecipitation and assay of its phosphorylating activity were negative (data not shown).

Experiments were also carried out in which Myc-CK2 β was fused to GST and GFP. These fusion constructs, when cotransfected with HA-CK2 α , were not able to cause the appearance of CK2 activity in the ectokinase domain (data not shown). Previous work (13) had demonstrated that GST-CK2 β fusion was able to bind CK2 α and stimulate its activity. These results indicate that although Myc-CK2 β can be exported, larger or different additions to the N-end can affect the export of this protein or interfere with the formation of the CK2 holoenzyme ectokinase.

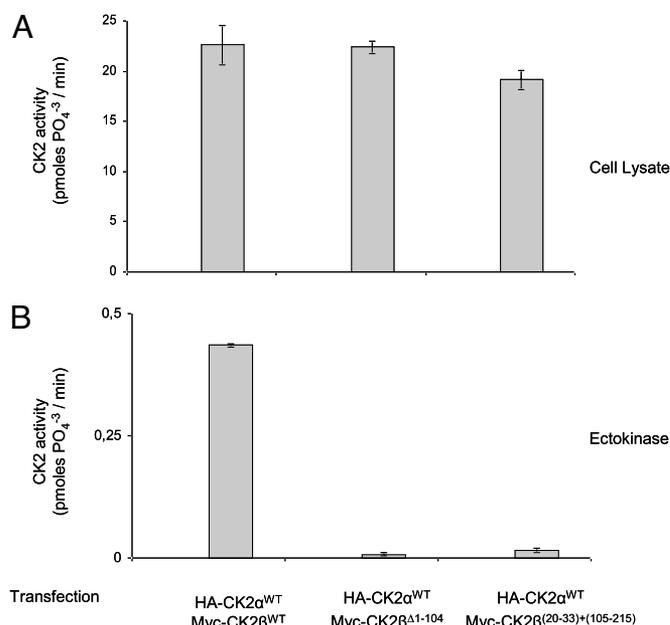


Fig. 5. CK2 activity using CK2 β fragment fusion construct. The CK2 activity present in anti-HA immunoprecipitates of cell lysates (A) and the shedding liquid (ectokinase) (B) was measured as described in *Materials and Methods*, using Myc-CK2 β ^{WT}, Myc-CK2 β Δ 1–104, and the Myc-CK2 β (20–33)+(105–215).

As mentioned previously, several authors (2–5) have provided evidence to indicate that the ectokinase activity of CK2 may have important physiological effects mediated through the extracellular phosphorylations of vitronectin, C9 complement protein, or the collagen XVII receptor. The findings described above indicated

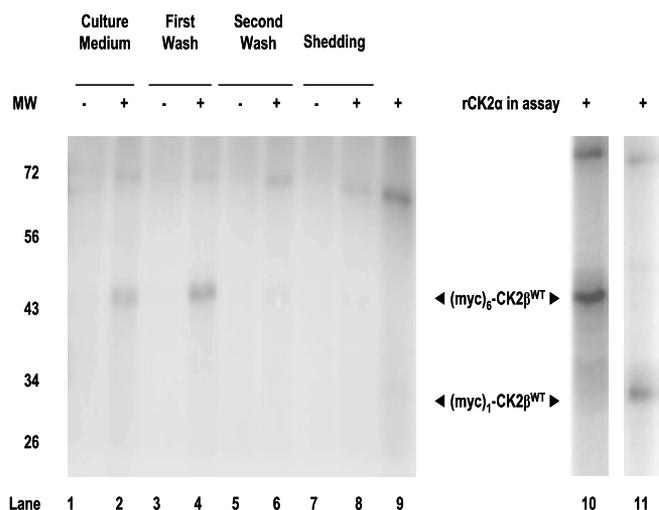


Fig. 6. The presence of free CK2 β in the culture medium. Free CK2 β was detected in cells transfected with (Myc) $_6$ CK2 β and (Myc) $_1$ CK2 β through immunoprecipitation and autophosphorylation as described in *Materials and Methods*. The culture medium, two subsequent washes of cells, and the shedding liquid were immunoprecipitated with anti-Myc antibodies, and the immunoprecipitates were incubated with (+) and without (–) recombinant CK2 α and [γ -³²P]ATP to cause autophosphorylation of CK2 β . The radioactive bands detected after SDS/PAGE of the immunoprecipitates through exposure in a Phosphorimager are shown. In lane 10, the analysis was carried out in the culture medium of cells transfected with (Myc) $_6$ CK2 β ^{WT}, and in lane 11, the analysis of the culture medium was carried out with cells transfected with (Myc) $_1$ CK2 β ^{WT}. The arrows show the positions of the (Myc) $_1$ CK2 β ^{WT} and (Myc) $_6$ CK2 β ^{WT}.

that CK2 ectokinase activity depends on the concurrent availability of CK2 β to bind the catalytically active CK2 α . Therefore, the physiological effects of the CK2 ectokinase may be regulated through the synthesis of CK2 β or through factors that may interfere with CK2 β binding to CK2 α . On the other hand, previous work from several laboratories (14, 15) has provided evidence that cells sometimes have an unbalanced stoichiometry of CK2 α and CK2 β . Cells that contain an excess of CK2 β over CK2 α subunits would export the regulatory subunit to the external medium. Free CK2 β has been shown to interact with several other protein kinases such as A-Raf, c-Mos, and Chk1 (16). These findings induced us to postulate that CK2 β could act as a wild-card regulator of kinases (17). It is pertinent therefore to ask whether these other kinases can also be carried out of cells by this regulatory subunit and by this mechanism they may function as exo or ectokinases. CK2 β has also been shown to interact with a large number of other proteins, many of which are substrates of CK2 (21). It is possible that these proteins may also be exported through the binding of CK2 β .

Materials and Methods

CK2 β Mutants. The CK2 β deletion mutants were produced by PCR. The cDNA of CK2 β from *Xenopus laevis* cloned in pCEFL-HA was used as template. The forward primers used added an EcoRI restriction site: E09F, 5'-TAT ATA GAA TTC ATG GAT TAG CTG G-3' for Myc-CK2 β ^{Δ1-8}; E20F, 5'-TAT ATA GAA TTC AGA GTT TTT CTG TGA G-3' for Myc-CK2 β ^{Δ1-19}; E33F, 5'-TAT ATA GAA TTC AAA GTT TAA CTT GAC AG-3' for Myc-CK2 β ^{Δ1-32}; E50F, 5'-TAT ATA GAA TTC ACT GGA CAT GAT ACT G-3' for Myc-CK2 β ^{Δ1-49}; and E80F, 5'-TAT ATA GAA TTC ATA TGG ACT AAT CCA TG-3' for Myc-CK2 β ^{Δ1-79}. The sequence of the reverse primer was X215R (5'-TAT ATA CTC GAG TCA ACG CAT GGT C-3'), which added an XhoI restriction site. The amplified fragments were digested and subcloned in pCS2+MT vector, which yielded six Myc epitope-tagged ectopic proteins, digested with EcoRI and XhoI restriction enzymes. The substitution mutants Myc-CK2 β ^{F21F22/A} and Myc-CK2 β ^{DED/A} were produced with the QuikChange XL Site-Directed Mutagenesis Kit from Stratagene as described (18). The following pair of complementary primers were used: forward, 5'-GGT TTA AGA GGC AAT GAG GCT GCC TGT GAG GTG GAT G-3', reverse, 5'-CAT CCA CCT CAC AGG CAG CCT CAT TGC CTC TTA AAC C-3' for Myc-CK2 β ^{F21F22/A} and forward, 5'-CTG TGA GGT GGC TGC AGC CTA TAT CCA GGA TAA G-3', reverse, 5'-CTT ATC CTG GAT ATA GGC TGC AGC CAC CTC ACA G-3' for Myc-CK2 β ^{DED/A}. The fragment fusion in Myc-CK2 β ²⁰⁻³³⁺¹⁰⁵⁻²¹⁵ was produced with two primers: E20F/Q33R (5'-GCC AAA ATC CTT ATC CTG GAT ATA G-3') and X215R/Q50F (5'-CAG GAT AAG CTG GAC ATG ATA C-3') to amplified the fragments CK2 β ²⁰⁻³³ and CK2 β ¹⁰⁵⁻²¹⁵, respectively. Through the complementary sequence between the primers Q33R and Q50F the amplified fragments formed a template that was used for a PCR with the primers E20F and X215R. This amplicon was digested with EcoRI and XhoI restriction enzymes and subcloned in pCS2+MT vector digested with the same restriction enzymes. The construct pRc-CMV+CK2 β ^{WT}, which produce a one Myc epitope-tagged CK2 β ^{WT} subunit, was kindly provided by David Litchfield (Western Ontario University, Ontario, Canada).

Transfection of HEK-293T Cells. HEK-293T cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) at 37°C with 5% CO₂. Subconfluent HEK-293T cells were transfected (or cotransfected) with the cDNA coding for the CK2 α subunit of *X. laevis* (6) cloned in pCEFL-HA and/or with the cDNA for CK2 β of *X. laevis* (mutant or wild type) cloned in pCS2+MT, which generates expressed CK2 α with one HA and CK2 β with six Myc epitope tags, respectively. Before transfection, the complete medium was withdrawn and replaced with transfection medium (DMEM without FBS and antibiotics). The vector constructs (4 μ g of DNA) and Lipofectamine (Invitrogen) were diluted in transfection medium, and transfection was carried out for 4 h. Transfection medium was replaced with complete medium, and the transfected cells were further incubated for 20 h.

Isolation of Ectopically Expressed CK2 Subunits in the Ectokinase Locus. The method of Kubler *et al.* (1) was used for the release of CK2 ectokinase from transfected cells by substrate-induced shedding of the enzyme. Briefly, subconfluent monolayer cell cultures in p60 plates (Falcon) were washed twice with buffer P (70 mM NaCl/30 mM Tris-acetate, pH 7.2/5 mM magnesium acetate/5 mM K₂HPO₄/0.5 mM EDTA/75 mM glucose) prewarmed at 37°C; the washes were preserved in ice when it was required. Cells were then incubated with 1 mg/ml phosvitin (Sigma) in 1 ml of buffer P per plate. The cells were incubated with phosvitin for 15 min at 37°C with mild shaking (shedding process). Subsequently, the extracellular liquid containing the ectoCK2 was aspirated and centrifuged at 2,000 \times g at 4°C for 5 min, and the supernatant fluid was then used for immunoprecipitation.

Preparation of Cell Lysates for Immunoprecipitation. After the shedding, HEK-293T cells were lysed to assay for ectopic CK2 levels in the cells. Briefly, cells were treated with ice-cold lysis solution containing PBS with 0.1% Nonidet P-40 (Sigma) and 10 μ l/ml protease inhibitor mixture (Calbiochem) for 30 min at 4°C (350 μ l of lysis solution per p60 plate). The resulting lysate was centrifuged at 14,000 \times g for 15 min in the cold, and the supernatant was kept on ice for immunoprecipitation. Protein concentration of lysates was determined by the Bradford method (19).

Immunoprecipitation. HA- or Myc epitope-tagged proteins in cell lysates (350 μ l), the culture medium (3 ml), the posterior washes (750 μ l), and the extracellular fluid obtained by the shedding procedure (1 ml) were incubated with 2 μ g of monoclonal anti-HA (F-7) or anti-Myc (9E10) antibodies (Santa Cruz Biotechnology) overnight at 4°C, then with 20 μ l of protein A Agarose (Sigma) for 4–5 h at 4°C. After centrifugation at 14,000 \times g for 1 min, the precipitates from the lysate and extracellular fluid were washed five times in buffer P, and the wash liquids were discarded.

Free CK2 β Detection Through Autophosphorylation. Using cells transfected with pCS2+MT+CK2 β ^{WT} or pRc-CMV+CK2 β ^{WT}, immunoprecipitates were prepared, as described above, using anti-Myc antibodies with the culture medium, two subsequent washes, and the shedding extracellular fluid. Ten microliters of these immunoprecipitates was incubated with 15 μ l of kinase buffer including 100 μ M NaCl, 0.3 pmol of recombinant His₆-CK2 α ^{WT}, and 10 μ M [γ -³²P]ATP (specific activity 60,000 cpm/pmol). Incubation was for 20 min at 37°C and stopped with 15 μ l of denaturing Laemmli solution with 7 M urea. The reactions were resolved in 12.5% SDS/PAGE gels and exposed in a Phosphorimager.

Assays for CK2 Activity in Immunoprecipitates. For CK2 assay, immunoprecipitates (20 μ l) were resuspended in 20 μ l of kinase buffer (50 mM Hepes, pH 7.5/10 mM MgCl₂/0.5 mM DTT), of which 5 μ l was used for assay. Assays contained in a total volume of 30 μ l included 100 mM NaCl, 150 μ M of the CK2-specific substrate peptide (RRADSDDDD) (Calbiochem), and [γ -³²P]ATP. In the case of the lysates, the specific activity of the ATP was 700 cpm/pmol, and the concentration was 100 μ M. In the case of the extracellular liquid, specific activity was 3,000 cpm/pmol, and the concentration was 20 μ M. Incubations were for 20 min (lysates) and 30 min (extracellular fluid) at 37°C. The reactions were processed according to the standard assay described in ref. 10.

Western Blots. Lysates of transfected cells prepared as described above (\approx 15 μ g of protein) were treated with Laemmli denaturing buffer containing 2-mercaptoethanol and heated at a 100°C for 3 min followed by 10% SDS/PAGE. In the case of proteins immunoprecipitated with anti-HA antibodies, the precipitates were dissolved in an equal volume of Laemmli denaturing buffer containing 2-mercaptoethanol and 7 M urea and heated at 100°C for 10 min before submitting to 10% SDS/PAGE. In both cases proteins in the gels were transferred to Immobilon membranes (Millipore) and probed with mouse monoclonal anti-Myc(9E10) antibody.

ACKNOWLEDGMENTS. We thank Dr. Catherine C. Allende and Dr. Lorenzo A. Pinna for many useful suggestions. This work was supported by a grant from Fondo Nacional de Desarrollo Científico y Tecnológico-Chile to Project 1060107 (to J.E.A.) and a Comisión Nacional de Investigación Científica y Tecnológica doctoral fellowship (to F.A.R.).

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