Role of the carboxyl terminus on the catalytic activity of protein kinase CK2α subunit

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Received 18 September 2002; revised 7 October 2002; accepted 7 October 2002

First published online 16 October 2002

Edited by Hans Eklund

Abstract Protein kinase CK2 (also known as casein kinase 2) has catalytic (α, α′) and regulatory (β) subunits. The role of carboxyl amino acids in positions from 324 to 328 was studied for *Xenopus laevis* CK2α. Deletions and mutations of these residues were produced in recombinant CK2α, which was assayed for kinase activity. Activity dropped 7000-fold upon deletion of amino acids 324–328. The key residues are isoleucine 327 and phenylalanine 324. A three dimensional model of CK2α indicates that these hydrophobic residues of helix αN may interact with hydrophobic residues in helix αE which is linked to the catalytic center.

Key words: Casein kinase 2; Deletions; Mutations; Protein structure; Protein kinase domain

1. Introduction

The protein kinases of eukaryotes constitute a large superfamily of enzymes that are involved in the regulation of the most crucial processes of cellular metabolism, growth and differentiation. Among the most fascinating aspects of the study of these enzymes are the wide variations on a common theme that are found in relation to the diversity of regulatory mechanisms that influence the specificity and activity of the catalytic domains that have common structural features. Hanks and Hunter [1] conducted pioneering work on the characterization and classification of protein kinases through the analysis of the amino acid sequences in the catalytic domain of these proteins. They identified 12 conserved sequences in these proteins that have served to define the kinase domain and to recognize many new kinases as genome sequences have been elucidated. The resolution of the crystal structure of the cAMP-dependent protein kinase [2], was followed by the solution of the structure of many other protein kinases, providing us with a great deal of information as to the general structural features of these enzymes as well as to the specific characteristics that explain the great versatility of their function and regulation [3].

Many protein kinases have sequences that extend, in both the amino and carboxyl ends, far beyond the catalytic domain defined by the 12 conserved sequences of Hanks and Hunter [1]. This is the case with the catalytic subunit of protein kinase CK2 (also known as casein kinase 2). CK2 is a pleiotropic enzyme, ubiquitous in eukaryotes, that has catalytic (α and α′) and regulatory subunits (β) that are usually conforming heterotetramers with the composition: $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$ [4–6]. The length and amino acid sequence of the extension beyond the protein kinase domain of the catalytic subunits of CK2 are in general highly conserved with the exception of the subunit of *Theileiria parva* (Fig. 1A,B). The conservation of the amino terminal extension is probably due to the fact that this portion of the molecule interacts with the activation loop and maintains the catalytic pocket in an open conformation [7]. In the case of the *T. parva* enzyme, the amino terminal extension is longer, including 98 amino acids that contain a possible transmembrane sequence [8].

In contrast, there is greater variation in the carboxyl region of CK2α that extends beyond amino acid 324, which is the last conserved residue of the kinase domain. As seen in Fig. 1A, human and other vertebrate CK2α subunits possess 391 amino acids, including 67 amino acid residues extending beyond the kinase domain. The C-terminus of the α′ subunit is shorter having only 25 residues beyond the kinase domain. In *Drosophila*, *T. parva*, yeast, and in plants (*Zea mays*, *Arabidopsis thaliana*, and *Nicotiana tabacum*) the carboxyl regions are much shorter, the extension being 5–10 amino acids beyond the kinase domain.

In our laboratory, we have asked what is the role of the carboxyl terminus of CK2α in relation to catalytic activity and what is the minimal extension required to maintain that activity. The present report describes the kinase activity obtained with a series of deletion and replacement mutants that involve the region between amino acids 323 and 328. The results point to a key role for phenylalanine 324 and isoleucine 327 in maintaining the activity of the enzyme. In a structural model of CK2α from *Xenopus laevis*, it can be seen that these residues, which are located in helix αN, interact hydrophobically with amino acid side chains of helix αE, which is in turn involved in maintaining the appropriate structure of the catalytic center of the enzyme.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (6000 Ci mmol^{-1}) and glutathione–Sepharose were purchased from Amersham Pharmacia Biotech. Ni^{2+}-NTA-agarose was from Qiagen. The protease-inhibitor cocktail, monoclonal antibodies anti-His, anti-CK2α and anti-CK2β were from Calbiochem. β-Casein, anti-GST monoclonal antibody, and the synthetic peptide substrate were purchased from Sigma-Aldrich. Pfu DNA polymerase was from

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0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)03555-X

MBI Fermentas. BioMax MS film and BioMax Transcreen HE intensifying screen were from Kodak.

2.2. Generation of CK2α mutants

CK2α mutants were constructed using cDNA from *X. laevis* cloned in *Bgl*II and *Not*I restriction sites of pT7HX vector. This plasmid contains a 6×histidine-tag and a recognition sequence for Factor Xa immediately before the multicloning site. All mutant CK2α subunits were amplified by PCR using Pfu DNA polymerase. Deleted mutants were prepared using the forward primer 5′-CGACTCACTATAGGGAGACCAC-3′ (HXFOR) and the following reverse primers: 5′-TATATAGCGGCCGCTGGTCCTTATACGATGGG-3′ ($CK2\alpha^{1-328}$), 5′-ATATATGTCGACTTAGATGGGATAGAAGTATG-3′ ($CK2\alpha^{1-327}$), 5′-ATATATGTCGACTTAGGGATAGAAGTATGG-3′ ($CK2\alpha^{1-326}$), 5′-ATATATGCGGCCGCTACGATGGGATTAGAAGTATGG-3′ ($CK2\alpha^{1-324}$), 5′-TATATAGTCGACTTAGTATGGGTGGTCCATAGC-3′ ($CK2\alpha^{1-323}$), and 5′-ATATATGTCGACCTAAGCGTATGGGTGGTC-3′ ($CK2\alpha^{1-324}$F324A).

Full-length CK2αI327G site-directed mutant was obtained using a standard PCR overlapping method as published [9], using forward primer HXFOR and reverse primer 5′-TTTGACAGCTTATCATCGATAACGT-3′ (HXREV), together with the mutagenic primers: 5′-CTTCTATCCCGGCGTAAAGGACC-3′ (328FOR); 5′-GGTCCTTTACGCCGGGATAGAAG-3′ (328REV).

Amplified CK2α mutants were subcloned in *Bgl*II and *Not*I or *Sal*I sites of the pT7HX vector. Design of the GST-$CK2\beta^{S2,3G}$ construct has been published [10]. All constructs were confirmed by automatic sequencing using an ABI Prism DNA Sequencer (Perkin Elmer). In the figures and Table 1, an abbreviated nomenclature is used to indicate deletion (overall length indicated) and substitution (amino acid changes indicated) mutants.

2.3. Recombinant proteins

Expression of His-tag proteins in *Escherichia coli* strain BL21 (DE3)plysS and further purification by Ni^{2+}-NTA-agarose affinity chromatography was described [11]. GST-$CK2\beta^{S2,3G}$, a β form not phosphorylated by CK2α, was expressed in *E. coli* strain DH5α and affinity purification was performed using glutathione–agarose protocol recommended by the manufacturer. This non-phosphorylatable mutant of CK2β was used in order to circumvent the autophosphorylation of the holoenzyme, $CK2\alpha_2\beta_2$, thereby limiting the phosphorylated products to those of the added substrate. $CK2\beta^{S2,3G}$ has been shown to retain the same capacity to interact with and activate CK2α subunits as the wild-type CK2β [10]. Eluted proteins were analyzed using 12% SDS–PAGE, specifically identified using monoclonal antibodies and densitometrically quantified using bovine serum albumin as protein standard. Proteins were aliquoted and stored at −80°C.

2.4. CK2 assays and kinetic determinations

The standard assay for $CK2\alpha^{wt}$ and CK2α mutants and determinations of kinetic constants were carried out in 30 μl reaction volume containing 100 μM [γ-^{32}P]ATP (specific activity 1000–1300 cpm $pmol^{-1}$), 10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 50 mM NaCl, and either dephosphorylated casein or peptide RRRAADSDDDDD as substrate. 2 pmol of CK2α forms were used, either alone or, where mentioned, with an equimolar amount of the non-phosphorylatable regulatory subunit GST-$CK2\beta^{S2,3G}$ which reconstitutes the holoenzyme. In this latter case 100 mM NaCl was used for the assay. After 10 min incubation at 30°C, the mixture was spotted onto a P81 phosphocellulose filter which was then washed three times in cold 75 mM H_3PO_4. Filter-associated radioactivity was measured by scintillation counting. Activity determined using gel electrophoresis and autoradiography was carried out under the same conditions as above but with [γ-^{32}P]ATP specific activity 2000–3000 cpm $pmol^{-1}$. After incubation for 10 min at 30°C, an aliquot of the mixture was subjected to 12% SDS–gel electrophoresis and the phosphorylated β-casein band visualized by autoradiography. The extent of protein phosphorylation was evaluated by densitometric analysis. For thermal stability studies 2 pmol of holoenzyme was reconstituted and protein concentration was adjusted with bovine serum albumin. After preincubation for different times at 45°C, an aliquot of the mixture was deposited in an ice-cooled tube with assay mix. The residual activity was determined by paper or gel electrophoresis methods as mentioned above.

2.5. Modeling

The model for CK2α subunit from *X. laevis* was obtained using MODELLER group of programs and the crystal structure of the catalytic subunit of CK2α from *Z. mays* at 2.1 Å resolution as a template [12] (PDB code 1A60). Images were generated using Web Lab Viewer Pro Software (Molecular Simulations, Inc.).

3. Results

Fig. 1C shows a series of deletion and substitution mutations that were prepared in carboxyl region of the CK2α subunit, bordering the edge of the protein kinase domain as defined by the typical conserved sequence. These recombinant mutant proteins were expressed in *E. coli* with $(His)_6$ tags and purified on affinity columns.

The results of ^{32}P incorporation assays measured by the phosphocellulose paper method allowed us to quantify activity of the mutants in the absence of CK2β with deletions up to amino acid 326 ($CK2\alpha^{1-326}$). Some of mutants gave barely measurable or no activity in this assay. As seen in Fig. 2, deletion of 63 amino acids of the carboxyl tail to generate $CK2\alpha^{1-328}$ causes a drop of 45% of activity. This result is similar to that seen previously when 60 amino acids were deleted to generate $CK2\alpha^{1-331}$ [13]. Each single amino acid deleted thereafter, however, causes a proportionally larger drop in activity. Thus $CK2\alpha^{1-327}$ is less than 20% as active as $CK2\alpha^{1-328}$, and $CK2\alpha^{1-326}$ is 17 times less active than the deletion mutant that terminates with isoleucine 327. The importance of isoleucine 327 residue was verified by mutating it to glycine but in this case the change was made within the full-length enzyme. As shown in Fig. 2 and summarized in Table 1, full-length mutant CK2αI327G is 42 times less active than the full-length wild-type subunit and only four-fold more active than $CK2\alpha^{1-326}$ protein. Deletion of two more amino acids to generate $CK2\alpha^{1-324}$ resulted in a protein that has no activity as measured by the phosphocellulose paper assay. Mutant CK2α proteins were also assayed for casein phosphorylation in the presence of the CK2β regulatory subunit (Table 1) and stimulation of catalytic activity was in the nor-

Table 1
Activity and kinetic parameters of deletion and substitution CK2α mutants

CK2α subunit	Activity (pmol ^{32}P pmol α^{-1} 10^{-2})	CK2β stimulation (fold)	K_m ATP (μM)	K_m peptide (μM)	K_m casein (mg ml^{-1})	k_{cat} (min^{-1})
Wild-type	310	6	8.4	185	1.9	4.3
1–328	170	9.8	11.5	165	1.3	1
1–327	30	8.2	6.9	77	1	0.2
I327G	7.4	3.3	n.d.	n.d.	n.d.	n.d.
1–326	1.8	8	n.d.	n.d.	n.d.	n.d.

Activity was determined with 2 pmol His-CK2α subunits alone. CK2β stimulation was measured using 2 pmol GST-$CK2\beta^{S2,3G}$ mutant. Kinetic constants were obtained using 2.2 pmol His-CK2α forms, as described in Section 2. n.d., not determined. The results are representative of at least three separate determinations with a S.E.M. of less than 15%.

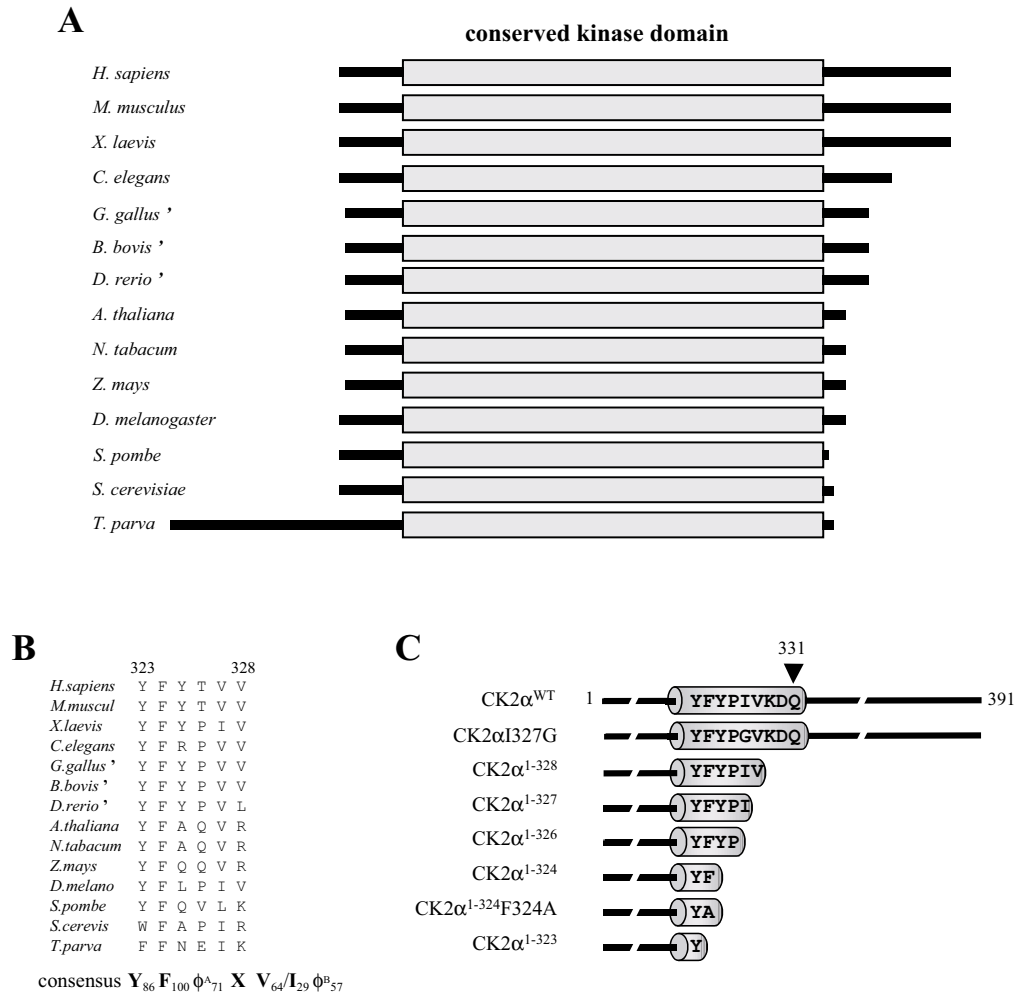

Fig. 1. Variations in the catalytic subunits of protein kinase CK2 of different species. A: Shown are protein kinase domains (gray boxes) and the relative lengths of the N- and C-terminal segments (black lines) of several CK2α subunits. B: Alignment of residues 323–328 for the same species as in A. The consensus sequence for these CK2α C-terminal regions is given below, where the subscript is the percentage use of the amino acid, X corresponds to any non-charged amino acid, ϕ^A is Y, A or L, and ϕ^B is V or L. C: A schematic view of the deletion and point mutations in CK2α from *X. laevis* used in this work.

mal range of four- to nine-fold. For these studies, the use of the non-phosphorylatable CK2β subunit, which has nearly identical properties as the unmodified CK2β [10], facilitated the measurement of the stimulatory effect. The possible effect of the GST moiety of this chimeric subunit has been considered in previous studies from this laboratory [10,14] and shown to have negligible effect on interaction and no effect on activation of CK2α.

Kinetic parameters were determined for those CK2α mutant subunits that maintain sufficient activity and stability for accurate measurement (Table 1). It is clear that these mutants have very similar $K_{m_{app}}$ values for ATP, for casein and model peptide substrate. However, the k_{cat} value for these mutants decreased considerably with the successive deletion of terminal amino acids.

In order to detect CK2α activity with the less active mutants, and to estimate activity when assayed in the presence of CK2β, phosphorylated substrate casein was detected using SDS–polyacrylamide gels. For this purpose, the specific activity of the [γ-^{32}P]ATP and autoradiographic exposure times were increased.

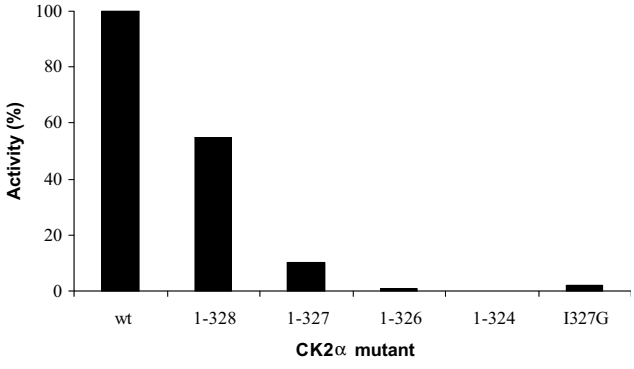

Fig. 2. The effect of carboxyl end deletions and the mutation of isoleucine 327 on the catalytic activity of CK2α. Enzymatic activity was measured by the phosphocellulose filter method using 5 mg ml^{-1} casein as substrate. The maximal activity for the wild-type holoenzyme corresponds to 3.1 pmol ^{32}P pmol α^{-1} min^{-1}. The results are representative of at least three separate determinations with a S.E.M. of less than 15%.

Using these conditions (Fig. 3), it is possible to determine activity of CK2α$^{1-324}$ assayed in the presence of CK2β. Densitometric measurements show that CK2α$^{1-324}$ activity is seven-fold lower than CK2α$^{1-326}$. Deletion of phenylalanine 324 to generate CK2α$^{1-323}$ causes another dramatic drop of approximately 10-fold as seen by densitometry of the phosphorylated casein. The importance of this phenylalanine, which is the last conserved amino acid of the kinase domain, is again demonstrated by the fact that mutant CK2α$^{1-324}$F324A, in which this residue is replaced by alanine, has an activity that is four-fold lower than CK2α$^{1-324}$.

In order to determine how significant was the trace of catalytic activity observed in CK2α$^{1-323}$, its activity was compared to that of mutant CK2αD156A which has the essential catalytic aspartic acid residue mutated to alanine and which has been used as an inactive subunit [11]. It was not possible to observe casein phosphorylation with this mutant under the conditions described in Fig. 3A. However, with increased exposure time (Fig. 3B) distinct phosphorylation is observed when the CK2αD156A mutant was assayed in the presence of CK2β. The densitometric comparison indicates that CK2α$^{1-323}$ is 2.5-fold more active than mutant CK2αD156A. Since the activity of CK2α$^{1-326}$ was assayed by both methods, this deletion mutant was used to extrapolate from the filter paper assay to the densitometric quantification of gel autoradiograms. This extrapolation allows one to calculate that the activity of CK2α drops almost 7000-fold by the deletion of five amino acids from CK2α$^{1-328}$ to CK2α$^{1-323}$. The key residues in this short stretch seem to be isoleucine 327 and phenylalanine 324.

The deletions and mutations in this region of the carboxyl end of CK2α do not affect the binding of the CK2β subunit as evidenced by the fact that the regulatory subunit continues to stimulate catalytic activity in a normal range (Table 1). Activity measured in all these cases was inhibited almost completely by the addition of heparin in the assay (data not shown).

Thermal stability of deleted and mutated holoenzymes was measured by incubating each CK2α subunit in the presence of CK2β at 45°C for different times and subsequently assaying the capacity to phosphorylate casein. Fig. 4 presents the results obtained which indicate that step-wise deletion of amino acids 327–324 causes an increasingly greater loss in the thermal stability of the holoenzyme. Mutation of isoleucine 327 to glycine in the full-length enzyme causes a rather dramatic labelization of the enzyme structure as measured by its loss of heat stability when compared to the unmodified holoenzyme.

4. Discussion

When the sequences of the catalytic subunits of protein kinase CK2 are aligned, the great variability of the length of the carboxyl tail that extends beyond the highly conserved catalytic domain becomes evident. These subunits can be grouped into three classes: the vertebrate CK2α subunits that have carboxyl extensions of 60 or more amino acids; the vertebrate CK2α′ proteins that contain intermediate size carboxyl segments with approximately 25 residues; and the catalytic subunits of plants, insects and lower eukaryotes that have very short extensions 5–10 residues downstream of the last conserved amino acid of the kinase domain. Obviously, all of

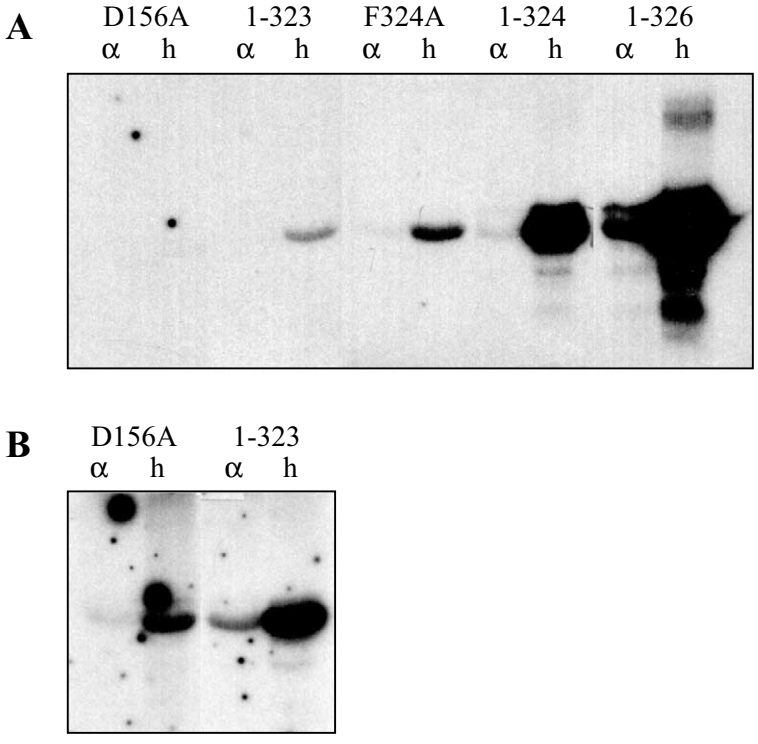

Fig. 3. Autoradiographic assay of the activity of CK2α deletion mutants. A: Phosphorylation of β-casein by 2 pmol of mutated α subunit alone (indicated α) or 2 pmol reconstituted mutated holoenzyme with an equimolar amount of GST-β^{S2,3G} (indicated h). Proteins were separated by SDS–PAGE, autoradiographed (1 h exposure time) and associated radioactivity determined by densitometric analysis. B: Overnight exposure of the same samples as depicted in A. The bands correspond to 8 μg β-casein. The results are representative of two or more different experiments with a S.E.M. of less than 18%.

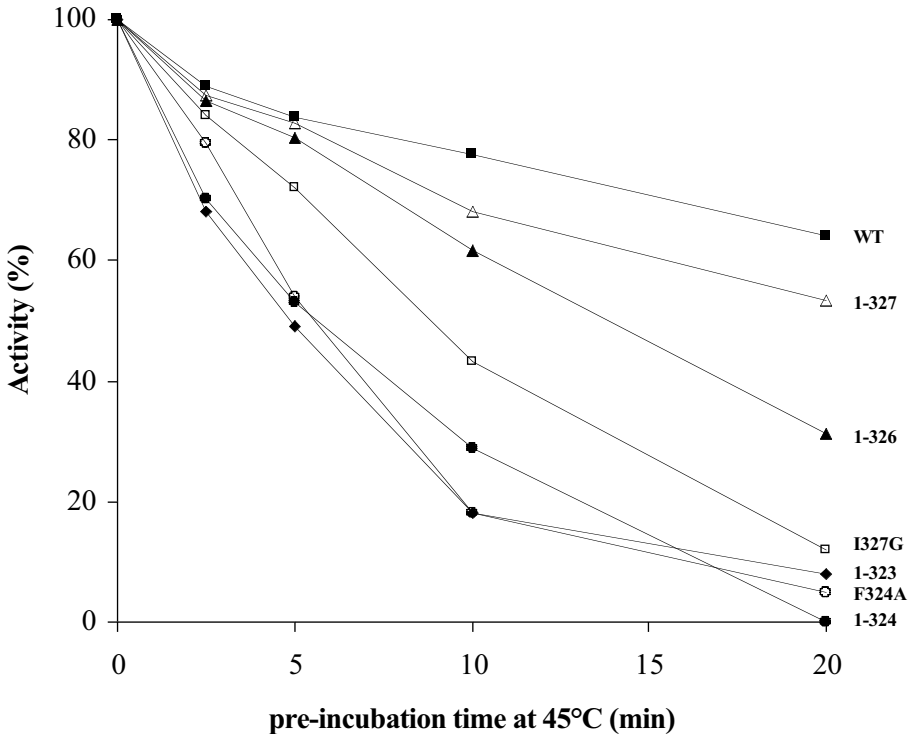

Fig. 4. Thermal stability of the CK2 holoenzyme, reconstituted with different deletion or substitution mutants of CK2α. Wild-type and mutant holoenzymes were reconstituted on ice by combining 2 pmol each His-CK2α subunit with an equimolar amount of GST-CK2$\beta^{S2,3G}$. Preincubation was at 45°C for the times indicated, followed by ice-cooling. The catalytic activity was determined at 30°C as described in Section 2. The results are representative of two to four different experiments with a S.E.M. of less than 21%.

these types of subunits maintain the catalytic activity of the protein required to fulfill the needs of the particular cell or organism. On the other hand, the carboxyl regions may serve regulatory or specificity functions for these enzymes. Recently, the laboratory of Litchfield [15] has explored the possible function of the carboxyl tail of mammalian CK2α and absent in CK2α′. These authors have presented evidence that the carboxyl extension of CK2α has sites phosphorylated by CDK's during mitosis that allow CK2α to interact with the protein pin-1. This modification of the carboxyl end, which

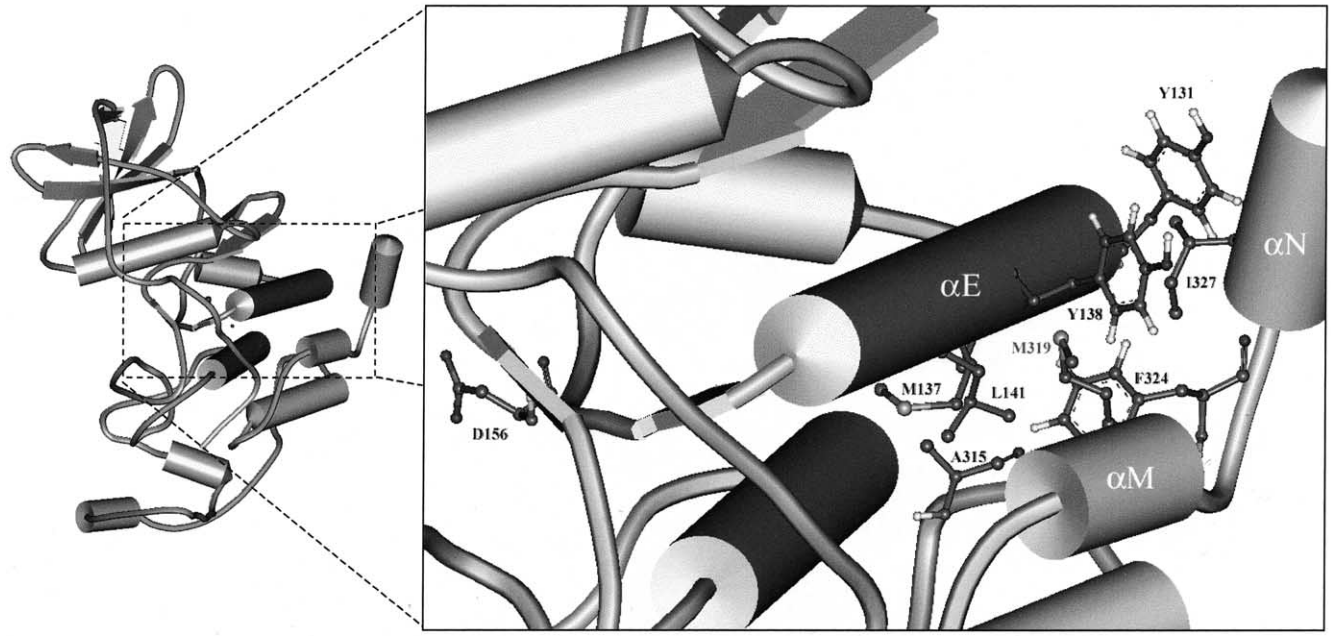

Fig. 5. Hydrophobic interactions stabilize the correct spatial position of aspartic acid 156 to confer activity to the CK2α. A model of the three dimensional structure of the *X. laevis* CK2α was constructed using the maize crystallographic structure as a template (left). The insert corresponds to a zoom of the hydrophobic pocket formed by the helices αE, αM and αN. It is shown that some residues probably interact to form a hydrophobic pocket and to maintain the spatial position of the catalytic aspartic acid 156. See details in the text.

does not occur in the α′ subunits, could explain why CK2α can phosphorylate some proteins at specific stages of the cell cycle. Alternatively, the carboxyl extensions of the longer catalytic subunits may contain 'docking sites' that allow these proteins to interact with specific substrates [16].

In the work presented above, a different question has been asked: what is the minimum length of the carboxyl extension required to maintain the catalytic activity of *X. laevis* CK2α? Although CK2α from *X. laevis* has a long carboxyl region, previous evidence indicated that this extension did not greatly influence catalytic activity since deletion of 60 amino acids to produce $CK2\alpha^{1-331}$ did not greatly reduce activity [13]. Also, with human CK2α, spontaneous proteolytic cleavage that removes 50 or more residues of the carboxyl tail of CK2α did not significantly affect activity and this truncated form was useful in the crystallization of the holoenzyme tetramer [17].

The results presented in this communication clearly show that a limited number of residues occupying positions 324–328, at the carboxyl border of the protein kinase domain of CK2α, play a very important role in maintaining the catalytic activity of this protein. Isoleucine 327 and phenylalanine 324 are especially critical in the maintenance of activity. In order to understand the structural basis for the importance of these residues in the catalytic function of the enzyme, a model of the three dimensional structure of the *X. laevis* CK2α was constructed based on the *Z. mays* crystallographic structure for the same protein [12]. This model shows that the particular residues of the carboxyl end which were studied here are situated in the helix αN. The hydrophobic side chains of the key residues phenylalanine 324 and isoleucine 327 appear to be in close contact with hydrophobic residues in helix αE, located in the middle of the CK2α structure and which extends across the whole carboxyl lobe (Fig. 5). In particular, isoleucine 327 may be sandwiched between the phenolic side chains of tyrosines 131 and 138 of the helix αE, while phenylalanine 324 seems to be buried in a hydrophobic pocket formed by the helices αE, αM and αN. The helix αE may play a central role in the activity of CK2α since, being in the heart of the structure, it heads into the catalytic loop which includes catalytic aspartic acid 156.

The decrease in heat stability of the enzyme that accompanies the removal of the amino acids that are important for catalysis agrees with the proposed role of these residues in maintaining the overall architecture of the active enzyme.

The almost universal conservation of phenylalanine located in the position 324 suggests that the role proposed for this amino acid in the case of CK2α may be also valid for other protein kinases. Inspection of the four amino acids following the phenylalanine demonstrates that, in general, hydrophobic residues are abundant, and that the amino acid corresponding to isoleucine 327 in *X. laevis* CK2α is very often replaced by valine. Conversely, the amino acids in helix αE that are observed to be in contact with isoleucine 327 of helix αN are also in most cases of a hydrophobic nature (tyrosine or phenylalanine) and are consistently separated by a stretch of six residues. If similar results were to be obtained with other protein kinases, one would have to consider extending the conserved protein kinase domain to include the hydrophobic residues that follow the highly conserved phenylalanine that corresponds to amino acid 324 in CK2α.

Acknowledgements: We gratefully acknowledge the useful discussions with Prof. Tom Blundell of the University of Cambridge. This work was supported by FONDECYT Project 1000624 to J.E.A. and FONDECYT Doctoral Project 2000066 to J.T. and by a Collaborative Research Initiative of the Wellcome Trust.

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