Tyrosine phosphorylation of protein kinase CK2 by Src-related tyrosine kinases correlates with increased catalytic activity

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Casein kinase-2 (CK2) is a pleiotropic and constitutively active serine/threonine protein kinase composed of two catalytic (α and/or α') and two regulatory β -subunits, whose regulation is still not well understood. In the present study, we show that the catalytic subunits of human CK2, but not the regulatory β -subunits, are readily phosphorylated by the Src family protein tyrosine kinases Lyn and c-Fgr to a stoichiometry approaching 2 mol phosphotyrosine/mol CK2 α with a concomitant 3-fold increase in catalytic activity. We also show that endogenous CK2 α becomes tyrosine-phosphorylated in pervanadate-treated Jurkat cells. Both tyrosine phosphorylation and stimulation of activity are suppressed by the specific Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine. By comparison, mutations giving rise to inactive forms of CK2 α do not abrogate and, in some cases, stimulate Lyn and c-Fgr-dependent tyrosine phosphorylation of CK2. Several

radiolabelled phosphopeptides could be resolved by HPLC, following tryptic digestion of CK2 α that had been phosphoradiolabelled by incubation with [³²P]ATP and c-Fgr. The most prominent phosphopeptide co-migrates with a synthetic peptide encompassing the 248–268 sequence, phosphorylated previously by c-Fgr at Tyr²⁵⁵ *in vitro*. The identification of Tyr²⁵⁵ as a phosphorylated residue was also supported by MS sequencing of both the phosphorylated and non-phosphorylated 248–268 tryptic fragments from CK2 α and by on-target phosphatase treatment. A CK2 α mutant in which Tyr²⁵⁵ was replaced by phenylalanine proved less susceptible to phosphorylation and refractory to stimulation by c-Fgr.

Key words: casein kinase-2, c-Fgr, Lyn, Src tyrosine kinase, tyrosine phosphorylation.

INTRODUCTION

Casein kinase-2 (CK2), a protein kinase, has been known for a very long time because of its constitutive activity and ability to phosphorylate acidic proteins such as casein and phosvitin *in vitro* (reviewed in [1]). CK2 holoenzyme is composed of two catalytic (α and/or α') and two regulatory β -subunits to form a heterotetramer, which can only be dissociated *in vitro* under denaturing conditions. The β -subunit appears to play a dual and, in some respects, enigmatic role by stimulating or inhibiting CK2 activity depending on the nature of the substrate and the experimental conditions employed [2–4]. One striking property of CK2 is its pleiotropic nature, which is documented by a growing list of substrates that at present exceeds 300 proteins. Phosphoacceptor sites for CK2 substrates are typically specified by multiple acidic residues downstream from the phosphorylatable amino acid, which is usually a serine or threonine [2,5] and in one case tyrosine [6]. Many of these proteins are participants in a variety of signalling pathways and other important cellular functions [2]. However, since numerous attempts to correlate the activity of CK2 itself to signals and/or second messengers have not yielded definitive insights into its regulation, it has been suggested that CK2 is an essential ‘house-keeping’ enzyme involved in the constitutive phosphorylation of a wide variety of protein targets. This possibility may be consistent with reports showing that abnormally elevated CK2 activity is observed frequently in tumours [4,7] and that CK2 is exploited

by many viruses to perform the phosphorylation of proteins essential for their life cycle [4]. Moreover, the catalytic subunits of CK2 have been shown to co-operate with proto-oncogenes in promoting cell transformation in various experimental models [8–12] and to display an anti-apoptotic effect in various transformed cell lines [13,14].

Based on these grounds, it is important to define the subtle molecular mechanisms that control CK2, and might tune the targeting of its wide spectrum of cellular substrates. Previously, it has been shown that the catalytic subunits of CK2, which are capable of phosphorylating tyrosine residues under special circumstances [6,15], can also undergo autophosphorylation at tyrosine residue(s) [16]. One of these autophosphorylation sites has been shown by mutational analysis to be Tyr¹⁸², a residue belonging to the activation loop, and also a structural element whose phosphorylation is required to achieve full activation of many protein kinases [17]. Since the amount of phosphate incorporated autocatalytically by CK2 at tyrosine residues is mostly substoichiometric, efforts to define the functional consequences of this event have been hindered. Nevertheless, it should be noted that the *in vitro* autophosphorylation of other kinases, extracellular-signal-regulated kinase in particular, mimics an event that is catalysed normally with much higher efficiency by heterologous kinases. By analogy, it therefore becomes possible that *bona fide* tyrosine kinases might phosphorylate the catalytic subunits of CK2 much more efficiently than CK2 itself. In a similar vein, it is notable that CK2 α

Abbreviations used: CK2, casein kinase-2; ESI, electrospray ionization; FSO₂BzAdo, *p*-fluoro-sulphonylbenzoyl-5'-adenosine; 4HCCA, α -cyano-4-hydroxy cinnamic acid; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PTK, protein tyrosine kinase; RP, reverse phase; TBB, 4,5,6,7-tetrabromobenzo-2-azabenzimidazole; TFA, trifluoroacetic acid.

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becomes tyrosine-phosphorylated on co-transfection of CK2 α and Abl tyrosine kinase into NIH-3T3 cells. [18]. This prompted us to investigate the possibility that the catalytic subunits of CK2 might undergo stoichiometric phosphorylation by protein tyrosine kinases (PTKs). Here we show that two tyrosine kinases of the Src family, Lyn and c-Fgr, phosphorylate readily both CK2 α and CK2 α' to a stoichiometry approaching 2 mol of phosphotyrosine/mol and that this phosphorylation correlates with a significant increase in CK2 catalytic activity. Surprisingly, however, the phosphorylated residues are not located in the activation loop, as in the case of autophosphorylation; rather phosphorylation affects several tyrosine residues, most of which have been mapped to Tyr²⁵⁵.

MATERIALS AND METHODS

Materials

[γ -³²P]ATP was purchased from Amersham Pharmacia Biotech (San Francisco, CA, U.S.A.), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) inhibitor and protease inhibitor cocktail were obtained from Calbiochem (Darmstadt, Germany) and Boehringer (Mannheim, Germany) respectively. Emodin, polylysine (46 kDa) and *p*-fluoro-sulphonylbenzoyl-5'-adenosine (FSO₂BzAdo) were purchased from Sigma (Dorset, U.K.); 4,5,6,7-tetrabromobenzo-2-azabenzimidazole (TBB) was synthesized according to the method described previously [19]. Sequencing-grade trypsin was purchased from Promega (Milano, Italy). The α -cyano-4-hydroxy cinnamic acid (4HCCA) matrix was purchased from Sigma. Anti-phosphotyrosine antibody was from ICN Biotechnology (Irvine, CA, U.S.A.). Anti-CK2 α C-terminal antiserum (directed against the residues 376–391 of human CK2 α) and anti-CK2 α (directed against the residues 66–86 of human CK2 α) were generated in our laboratory using keyhole limpet haemocyanin as a carrier. Other reagents were purchased from Sigma. All reagents used for MS were of HPLC grade.

Protein kinases

The CK2 α Y255F (Tyr²⁵⁵ $\rightarrow$ Phe) mutant was obtained using the 'QuikChange-Site Directed Mutagenesis' kit (Stratagene, La Jolla, CA, U.S.A.). Two synthetic oligonucleotide primers, 5'-GGGGACAGAAGATTTATTTGACTATATTGAC-3' and 5'-GTCAATATAGTCAAATAAATCTTCTGTCCCC-3' were used, each complementary to opposite strands of human α cDNA inserted into pT7-7 vector. Other CK2 α mutants, human wild-type CK2 α , CK2 α' , CK2 β and maize CK2 were expressed in *Escherichia coli* as described elsewhere [20–24]. Lyn, c-Fgr, Syk and Csk were isolated from rat spleen [25–28]. One enzyme unit was defined as the amount of tyrosine kinase that transferred 1 pmol of phosphate/min to 2 mM angiotensin II under standard conditions.

FSO₂BzAdo treatment

CK2 α was incubated at 30 °C in 50 mM Tris/HCl containing 1 mM FSO₂BzAdo and 10 mM MnCl₂. The reaction was stopped after 1 h by adding 40 mM 2-mercaptoethanol.

Phosphorylation of CK2 subunits by PTKs

The indicated amounts of CK2 subunits and CK2 α mutants were phosphorylated in 30 μ l of incubation medium, containing

50 mM Tris/HCl (pH 7.5), 5 mM MnCl₂, 30 μ M [γ -³²P]ATP (phosphorylation medium) and 10 units of PTKs. After 10 min of incubation, the reactions were stopped by addition of 2 % (w/v) SDS and the samples were subjected to SDS/PAGE. The degree of protein phosphorylation was evaluated either by analysis on a Packard (Meriden, CT, U.S.A.) Imager or by autoradiography and counting the identified radioactive bands. One unit was defined as the amount of enzyme that transferred 1 pmol of phosphate/min to 2 mM angiotensin II under standard conditions. Kinetic constants were determined by GraphPad Prism software fitting the data directly to the Michaelis–Menten equation using non-linear regression.

Peptide phosphorylation

c-Fgr activity towards the indicated amounts of peptide CK2 α (residues 248–268) and its derivative was assayed by incubating in phosphorylation medium for 10 min. Reactions were stopped by the addition of 1 mM HCl and processed as described in [29].

CK2 α activity towards the specific peptide RRRAADSDDDDD (30 μ M) was tested in the phosphorylation medium containing 5 mM MgCl₂ as described above. Reactions were terminated after 5 min of incubation by spotting 25 μ l of the mixture on P81 phosphocellulose papers and processed as described elsewhere [30].

In-gel tryptic digests

CK2 α (20 μ g) was phosphorylated in the absence of radiolabelled ATP as described above and loaded on to an SDS/polyacrylamide gel. Coomassie Blue-stained CK2 α was cut out of the SDS/polyacrylamide gel and in-gel-digested using established methods [31]. Briefly, the gel slices were destained overnight with 50 % (v/v) methanol/10 % (v/v) acetic acid. The gel slices were then washed three times for 10 min with water followed by two 10 min washes with 0.1 M NH₄HCO₃. Next, the gel slices were dehydrated and rehydrated with 100 % (v/v) acetonitrile and 0.1 M NH₄HCO₃ twice before drying down in a Speed Vac. The samples were then incubated with a solution containing 0.1 M NH₄HCO₃ and 55 mM dithiothreitol for 45 min at 56 °C followed by a 30 min incubation in a solution of 0.1 M NH₄HCO₃ and 10 mM iodoacetamide at room temperature (25 °C). After this treatment, the gel bands were dehydrated and rehydrated as described above, before drying in a Speed Vac. The dried gel pieces were rehydrated on ice for 45 min with 25 mM NH₄HCO₃ containing 0.025 μ g/ μ l sequencing-grade trypsin, followed by an overnight incubation at 37 °C. The tryptic peptides were extracted from the gel with four 15 min washes of 50 % acetonitrile/5 % (v/v) formic acid. The peptides were dried and resuspended in 5 μ l water.

MS of the tryptic peptides

Matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) spectra of phosphatase- and non-phosphatase-treated in-gel digests were examined on a Micromass MALDI–TOF mass spectrometer (Micromass, Wythenshawe, Manchester, U.K.) in positive-ion-reflector mode. Desalted tryptic peptides (1 μ l) were mixed with 4HCCA matrix solution [49.5 % acetonitrile/49.5 % (v/v) ethanol/1 % (v/v) trifluoroacetic acid (TFA); 1 μ l], spotted on to a MALDI-target plate and then allowed to dry at room temperature. Spectra were collected between a range of *m/z* 900 and 4000. The pulse voltage, the source voltage and the reflectron voltage were set at 3025,

15000 and 500 V respectively. The MALDI-TOF analyser was calibrated using angiotensin I, renin and adrenocorticotrophic hormone clip 18–39 in a ratio of 1:2:3. The spectra were analysed using MassLynx 3.5. ProFound (<http://prowl.rockefeller.edu/cgi-bin/ProFound>) was used to determine which of the peaks in CK2 α peptides were phosphorylated and non-phosphorylated.

The CK2 α sample was subjected to on-target phosphatase treatment using established methods [32]. Briefly, immediately after analysis by MALDI-TOF, the spotted sample was redissolved using 1.5 μ l of 50 mM NH_4HCO_3 containing 0.05 unit/ μ l alkaline phosphatase (Roche Biochemicals). The MALDI-target plate was placed in a humidified closed container and incubated for 30 min at 37 °C. The spotted samples were then reacidified with 0.5 μ l of 5 % TFA and the 4HCCA matrix was allowed to recrystallize at room temperature. The phosphatase-treated CK2 sample was re-analysed using the same conditions as above to determine whether the phosphorylated tryptic peptides had disappeared.

Nanospray/MS and MS/MS spectra of selected non-phosphorylated and phosphorylated CK2-tryptic peptides were obtained on a Micromass Q-ToF2 mass spectrometer (Micromass) using borosilicate tips (Econo 10, New Objective). Tryptic peptides from in-gel digests were desalted using C18 ZipTips (Millipore, Bedford, MA, U.S.A.) and concentrated samples (2–3 μ l) were loaded on to the borosilicate tips.

The TOF analyser was calibrated using an MS/MS spectrum of [Glu]-fibrinopeptide-B.

Survey and MS/MS spectra were acquired over a range of m/z 50–2000 using a cone voltage of 35 V and capillary of 1200 V to optimize spray. MS/MS spectra were acquired manually using collision energies ranging from 30 to 35 V. MS/MS spectra were processed by baseline subtraction and deconvoluted using the MaxEnt3 module of MassLynx 3.5. Peptide sequences were determined semi-automatically from the resulting, singly charged, deisotoped spectra using PepSeq version 3.3 supplied with MassLynx 3.5.

Resolution of radioactive CK2 α tryptic peptides by reverse phase (RP)-HPLC

CK2 α (5 μ g) was phosphorylated in the presence of [γ - ^{32}P]ATP, subjected to SDS/PAGE and blotted as described in the Materials and methods section. Radioactive bands corresponding to CK2 α were cut out of the filter and treated with trypsin (50 μ g/ml) for 4 h in 500 μ l of 0.1 M NH_4HCO_3 . The sample was then freeze-dried and washed exhaustively with water. Tryptic fragments were resuspended in 0.1 % TFA and subjected to analytical RP-HPLC using a Symmetry C18 column (46 mm $\times$ 250 mm; Waters, Milford, MA, U.S.A.), eluted at 1 ml/min with a linear gradient of acetonitrile containing 0.08 % TFA from 10 to 35 % for 110 min. The radioactivity of the fractions collected every 30 s was measured by liquid-scintillation counting.

Cell culturing, stimulation and lysis

The human leukaemia Jurkat T-cell line was maintained in RPMI 1640, supplemented with 10 % foetal calf serum, 10 mM Hepes, 2 mM L-glutamine, 100 units/ml penicillin and 100 g/ml streptomycin. The cells were stimulated by treatment (approx. 10×10^6 cells/ml) with 250 μ M pervanadate at 37 °C for 15 min. Cells were centrifuged and lysed for 15 min in ice-cold buffer containing 20 mM Tris/HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 10 % (v/v) glycerol, 1 mM Na_3VO_4 , 1 % (v/v) Nonidet P40 and, protease inhibitor cocktail.

The lysate was cleared by centrifugation at 12 000 g for 10 min (Eppendorf Microfuge 5415C).

Anti-CK2 α immunoprecipitation

Immunoprecipitation was performed with lysate corresponding to approx. 5×10^6 cells at 4 °C for 2 h with 2.5 μ l of anti-CK2 α C-terminal antiserum, followed by the addition of Protein A-Sepharose. Immunoprecipitates were washed twice with NET buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/5 mM EDTA/0.05 % (v/v) Nonidet P40/2 mg/ml BSA] and once with 50 mM Tris/HCl (pH 7.5). Samples were then loaded on to SDS/polyacrylamide (10 % gel), transblotted to Immobilon-P membranes and analysed by Western blotting by incubation with anti-phosphotyrosine antibody, followed by immunostaining with anti-CK2 α (66–86) antibody.

Immunostaining

Proteins transferred to nitrocellulose membranes were incubated with the indicated antibodies followed by the appropriate biotinylated secondary antibody and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS

Tyrosine phosphorylation of CK2 α by Src family PTKs

As shown in Figure 1(A), human CK2 α can be tyrosine-phosphorylated by two PTKs, Lyn and c-Fgr, of the Src family but not by Syk and Csk, which are not related to Src. The human CK2 α' subunit is also phosphorylated, whereas the regulatory β -subunit is not. The catalytic α -subunit of maize CK2 is phosphorylated by Lyn and c-Fgr, although less readily than its human homologue. As shown in Figure 1(B), phosphorylation of human CK2 α reached values approaching 2 mol phosphate/mol of either Lyn or c-Fgr. This result differs significantly from the tyrosine autophosphorylation of CK2, which remains largely substoichiometric even after prolonged incubation [16]. Based on these results, it is apparent that Lyn/c-Fgr target more than one tyrosine residue in CK2 α . It is probable, given the close similarities among kinases of the Src family, that other members of the family will also be capable of replacing Lyn and c-Fgr in the phosphorylation of CK2 α . This prediction is consistent with similar kinetic constants for phosphorylation of CK2 α calculated with either Lyn or c-Fgr. In particular, the low K_m values (0.68 and 0.76 μ M with Lyn and c-Fgr respectively) highlight the concept that CK2 α is indeed a good substrate for Src-related kinases.

Unlike tyrosine autophosphorylation [16], Lyn/c-Fgr-mediated CK2 α phosphorylation was prevented by the Src-specific inhibitor PP2 (see Figure 2A). Surprisingly, however, it is also prevented by emodin at a concentration that is inhibitory for CK2 α alone, but not for Src kinases. Similarly, the selective CK2 inhibitor, TBB [33], which is ineffective on Lyn/c-Fgr activity, prevented CK2 α phosphorylation by c-Fgr (Figure 2A) and Lyn (results not shown), as did prior irreversible inactivation of CK2 α by the ATP analogue FSO $_2$ BzAdo.

The possibility that tyrosine phosphorylation of CK2 α promoted by the addition of Lyn or c-Fgr could be accounted for by stimulation of CK2 α autocatalytic activity was ruled out by several lines of evidence. First, CK2 α mutants that are defective in catalytic and autophosphorylation activity (Δ 2–12, R191A/R195A/K198A, Y182F and Y188F) [16], were

Figure 1 Phosphorylation of CK2 subunits by different PTKs

(A) Anti-phosphotyrosine immunostaining of recombinant human (rh) CK2 α , CK2 α' and CK2 β -subunits, and recombinant maize (rm) α -subunit (200 nM) phosphorylated by the indicated non-receptor tyrosine kinases as described in the Materials and methods section. After incubation, the samples were subjected to SDS/PAGE, followed by transfer on to nitrocellulose and anti-phosphotyrosine immunostaining. The band slightly more mobile than CK2 α is generated by limited proteolysis of it as judged by immunoreaction with anti-CK2 α antibody. (B) Time course of CK2 α phosphorylation catalysed by Lyn (○) or c-Fgr (●). CK2 α (50 nM) was incubated with 20 nM Lyn or c-Fgr in the presence of [γ - 32 P]ATP as described in the Materials and methods section. The degree of protein phosphorylation was evaluated either by analysis on a Packard Imager or by autoradiography and counting of the identified bands. The results are representative of five different experiments.

phosphorylated by the Src kinases as efficiently as wild-type CK2 α or even more readily in some cases (Figure 2B). Secondly, CK2 α tyrosine phosphorylation promoted by Lyn and c-Fgr relies on Mn^{2+} as the activating cation, whereas Mn^{2+} inhibits tyrosine autophosphorylation [16]. Thirdly, in a similar vein, polylysine, which inhibits tyrosine autophosphorylation, [16] greatly stimulated CK2 α phosphorylation by c-Fgr (Figure 2A, lane 6) and Lyn (results not shown). On the other hand, association of CK2 α with equimolar amounts of the β -subunit to give the heterotetrameric holoenzyme, decreased the rate of Lyn/c-Fgr-mediated phosphorylation (Figure 2A, lane 7), but not as drastically as in the case of tyrosine autophosphorylation, which is almost abrogated entirely by association with the β -subunit [16].

It is therefore possible that ATP-binding site-directed compounds prevent tyrosine phosphorylation by hampering accessibility of tyrosine residue(s) located at or near the catalytic site. However, the expectation that the two tyrosine residues (Tyr¹⁸² and Tyr¹⁸⁸) in the activation loop might be the sole residue(s) phosphorylated by Lyn and c-Fgr was disproved by experiments using mutants where these residues were replaced by phenylalanines. Both these mutants, in which tyrosine autophosphorylation is either abolished or impaired [16], were in fact phosphorylated by c-Fgr as readily as wild-type CK2 α (Figure 2B). The same was observed with Lyn (results not shown).

Figure 2 Influence of various effectors (A) and mutations (B) on CK2 α tyrosine phosphorylation by c-Fgr

(A) Autoradiography of human wild-type CK2 α (100 nM) 32 P-phosphorylated by c-Fgr either in the absence (lane 1) or presence (lanes 2–7) of the following compounds: PP2 (10 μ M), emodin (20 μ M), TBB (40 μ M), FSO₂BzAdo (see pretreatment in the Materials and methods section), polylysine (PolyLys; 70 μ g/ml) and CK2 β (100 nM). Lane 8 shows c-Fgr autophosphorylation, as the assay was performed in the absence of CK2 α . (B) Autoradiography of wild-type (w.t.) CK2 α and its mutants (100 nM) phosphorylated by c-Fgr. The panels are representative of at least four different experiments.

Similar results were obtained by mutating two other tyrosine residues (Tyr²⁰⁹ and Tyr²¹¹), located just downstream from the activation loop, the former pointing into the active site in the crystal structure of CK2 α [34].

Likewise, a number of mutants lacking tyrosine residues located in the N-terminal segment (Tyr¹², Tyr²³ and Tyr²⁶) and in the β 1 strand (Tyr³⁹) were phosphorylated by Lyn and c-Fgr as, efficiently as, or even faster than, wild-type CK2 α (see Figure 2B).

Mapping the CK2 α residues which are phosphorylated by Src-related tyrosine kinases

In a further attempt to gain information about the CK2 tyrosine residues affected by Src kinases, the α -subunit of CK2 was phosphorylated exhaustively by [γ - 32 P]ATP in the presence of c-Fgr and subjected to tryptic digestion followed by RP-HPLC. As shown in Figure 3(A), several radioactive peaks were resolved, consistent with the concept that multiple sites are phosphorylated and the observed overall phosphorylation stoichiometry approaching 2 may reflect a more complex situation where more than two residues are partially phosphorylated.

Tryptic peptides derived from CK2 α that had been phosphorylated exhaustively by c-Fgr were also examined by MS. With MALDI-TOF analysis (results not shown), a peak with a mass consistent with a sequence corresponding to residues 248–268 was detected, as was a peak with a mass corresponding to a mono-phosphorylated form of this peptide. The latter peak was lost following on-target phosphatase treatment of the CK2 α -derived tryptic peptides (results not shown), an indirect indication that the peptide was phosphorylated. To extend the results obtained by MALDI-TOF using direct sequence analysis, tryptic

Figure 3 Resolution of tryptic phosphopeptides from ^{32}P -Tyr-CK2 α

(A) CK2- α ^{32}P -phosphorylated by c-Fgr was digested by trypsin and the radioactive peptides were resolved by RP-HPLC as described in the Materials and methods section. (B) The synthetic peptide reproducing the tryptic fragment 248–268 of CK2 α was phosphorylated by c-Fgr in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The phosphorylation mixture was then filtered through an RP-C18 resin (100 μl) and washed exhaustively with water containing 0.1% TFA. The anchored peptide was recovered by one-step elution with 500 μl of water/acetonitrile (40:60, v/v) solution and freeze-dried. The radioactive peptide was then resuspended in 0.1% TFA and subjected to RP-HPLC as in (A). The arrow denotes the elution time of the non-phosphorylated peptide.

peptides derived from phosphorylated CK2 α were analysed by electrospray ionization (ESI)–MS. By ESI–MS analysis of in-gel tryptic-digested CK2 α , phosphorylated previously by c-Fgr, non-phosphorylated and phosphorylated peptides corresponding to residues 248–268 were identified (Figure 4A). Based on the deconvoluted MS/MS spectra, the sequences of either the phosphorylated or non-phosphorylated tryptic peptides were determined revealing the phosphorylated residue within this tryptic peptide to be Tyr 255 . Identification of Tyr 255 as one of the phosphorylated residues was confirmed by preparing a synthetic peptide reproducing the 248–268 sequence of CK2 α , as well as another synthetic peptide where its first tyrosine, equivalent to Tyr 255 , was replaced by phenylalanine. Whereas the latter peptide was refractory to phosphorylation by c-Fgr, the wild-type peptide is phosphorylated readily with a $K_m = 31.8 \mu\text{M}$ (results not shown). Once phosphorylated and subjected to HPLC, the 248–268 peptide co-eluted with the main phosphoradiolabelled peptide generated by tryptic digestion of CK2 α (Figure 3B). These results prompted us to generate a CK2 α mutant in which Tyr 255 was replaced by phenylalanine. As shown in Figure 2(B), this mutant was phosphorylated by c-Fgr less readily than wild-type CK2 α (cf. lanes 1 and 11). The same was found using Lyn as the phosphorylating agent (results not shown). Taken collectively, these results indicate that Tyr 255 represents a major site of phosphorylation by Src kinases, accounting for a substantial amount of the phosphate incorporated. Apparently, other tyrosine residues are also phosphorylated by c-Fgr and Lyn, albeit to a lower stoichiometry, a circumstance that has hindered their identification.

Increases in catalytic activity of CK2 α on phosphorylation by Src PTKs

In an attempt to determine whether tyrosine phosphorylation of CK2 α might affect its catalytic activity, aliquots of CK2 α were preincubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ either in the absence or presence of c-Fgr; the amount of phosphate incorporated was monitored after SDS/PAGE of an aliquot of the samples. At the end of preincubation, each sample was assayed for its catalytic activity towards a peptide substrate specific for CK2. The results are shown in Figure 5(A): it can be seen that tyrosine phosphorylation of CK2 α is paralleled by a substantial increase in catalytic activity. Both tyrosine phosphorylation of CK2 and the c-Fgr-dependent increase in catalytic activity are reduced drastically if the specific Src inhibitor PP2 is added during preincubation. Note that the CK2 α mutant Y255F is less susceptible to tyrosine phosphorylation and, more importantly, is not significantly stimulated by incubation with c-Fgr (Figure 5B). A similar behaviour is displayed by maize CK2 α (Figure 5C), in which Tyr 255 is replaced by an asparagine.

Increased catalytic activity of human CK2 by c-Fgr-mediated tyrosine phosphorylation is also reflected by increased serine phosphorylation of its β -subunit. As shown in Figure 6, such an autocatalytic process is enhanced greatly if the α -subunit is preincubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and c-Fgr before the addition of equimolar amounts of the β -subunit to reconstitute the holoenzyme. Again, the cause–effect relationship between tyrosine phosphorylation of CK2 α and its increased autocatalytic activity is supported by the ability of the Src-specific inhibitor PP2 to prevent both events.

Endogenous CK2 α becomes tyrosine-phosphorylated in Jurkat cells

As shown in Figure 7, CK2 immunoprecipitated from quiescent Jurkat cells is not tyrosine-phosphorylated to any appreciable extent, as judged from Western blots performed with anti-phosphotyrosine antibody (Figures 7A and 7B, lanes 1). By comparison a modest, but reproducible, phosphotyrosine signal was detected if the catalytic subunit of CK2 was immunoprecipitated from cells treated previously with pervanadate to prevent phosphotyrosine dephosphorylation (Figures 7A and 7B, lanes 2). The tyrosine-phosphorylated CK2 α band underwent a mobility shift (Figure 7B, lane 2) similar to the one observed on *in vitro* tyrosine phosphorylation of recombinant CK2 α by c-Fgr (Figure 7B, lane Ctrl) and clearly represents only a minor proportion of the CK2 α present. Prime candidates for performing such a tyrosine phosphorylation of endogenous CK2 in living haematopoietic cells are the PTKs of the Src and Syk families, both of which become activated on pervanadate treatment (see e.g. [35,36]). The involvement of the former would be consistent with the observation that Lyn and c-Fgr, both members of the Src family, but neither Syk nor Csk, readily phosphorylate the recombinant catalytic subunits of human CK2 (either α or α') *in vitro* (see Figure 1A).

DISCUSSION

The results show that the catalytic subunits of protein kinase CK2 (α and α') are readily tyrosine-phosphorylated *in vitro* by two members of the Src family PTKs, Lyn and c-Fgr, to an overall stoichiometry approaching 2 mol phosphate/mol protein, with a concomitant 3-fold increase in CK2 catalytic activity. Both tyrosine phosphorylation of CK2 α and stimulation of catalytic activity are abrogated by the specific Src kinase inhibitor PP2. Unexpectedly, tyrosine phosphorylation of CK2 α by Lyn and

Figure 4 MS of *in vitro* phosphorylated CK2 α

Coomassie Blue-stained gel slices of CK2 α were subjected to in-gel tryptic digestion according to the Materials and methods section. An aliquot of the digested peptides was analysed on ESI-MS. Once CK2 tryptic peptides were identified using Profound, sequencing was performed on the peptides to determine which tyrosine residue was phosphorylated. **(A)** Raw data ESI-MS of *in vitro* phosphorylated CK2 showing non-phosphorylated and phosphorylated peptides corresponding to CK2 residues 248–268. Expected m/z values are 848.76 (observed 848.72) and 875.41 (observed 875.37) for the triply charged ions of the non-phosphorylated and phosphorylated species respectively. Some mass assignments of peaks were removed for high clarity of the Figure. **(B)** Deconvoluted MS/MS of the tryptic peptide corresponding to CK2 residues 248–268 (non-phosphorylated). The y-ion series shows the sequence to be VLGTEDLYDYLDKYNLELDPR. The spectrum shows peaks corresponding to monoisotopic MH^+ species. **(C)** The spectrum in **(B)** is expanded due to the abundance of species at m/z 272.18, which corresponds to fragmentation between proline and aspartic residues. **(D)** Deconvoluted MS/MS of the tryptic peptide corresponding to CK2 residues 248–268 (phosphorylated). The y-ion series shows the sequence to be VLGTEDLpYDYLDKYNLELDPR. The spectrum shows peaks corresponding to monoisotopic MH^+ species. **(E)** Spectrum in **(D)** is expanded due to abundance of species at m/z 272.18, which corresponds to fragmentation between proline and aspartic residues.

c-Fgr is also prevented by pretreatment of CK2 α with the ATP analogue FSO₂BzAdo and by the specific-CK2 inhibitor TBB, which is entirely ineffective on Src activity. A possible explanation for these observations could be that the tyrosine residue(s) affected

by Lyn and c-Fgr are located in the proximity of the catalytic site where the presence of ligands would hamper their accessibility. However, mutational analysis has ruled out any major involvement of the two tyrosine residues in the activation loop, of which

Figure 5 Variable stimulation by c-Fgr of the catalytic activity of human CK2α wild-type (A), Y255F mutant (B) and of maize CK2α (C)

Human CK2α (100 nM) wild-type (A), human Y255F mutant (B) and maize CK2α (C) were preincubated in 50 μl of basal medium for 20 min, either alone (control) or in the presence of active (+c-Fgr) or inactive c-Fgr (+c-Fgr and 5 μM PP2). An aliquot of each sample (20 μl) was then subjected to SDS/PAGE and the amount of P_i incorporated was quantified as described in the Materials and methods section. Another aliquot (25 μl) was supplemented with 5 mM MgCl₂ and CK2 activity was tested using the specific peptide substrate RRAADSDDDD. Tyrosine phosphorylation is expressed as pmol of P_i incorporated/mol of CK2α (hatched bars), and CK2 activity is expressed relative to the control incubated without c-Fgr (100%) (solid bars).

Figure 6 Tyrosine phosphorylation of CK2α promotes increased autophosphorylation of CK2 holoenzyme at its β-subunit

CK2α (100 nM) was preincubated in the phosphorylation medium (see the Materials and methods section) for 20 min either alone (lane 1), in the presence of c-Fgr (lane 2) or with c-Fgr and 5 μM PP2 (lane 3). The samples were then supplemented with 5 mM MgCl₂ and 100 nM CK2β and incubated for 10 min before SDS/PAGE and autoradiography.

Figure 7 Tyrosine phosphorylation of CK2α subunit in Jurkat cells

Cells treated without (lanes 1) and with (lanes 2) pervanadate were lysed and immunoprecipitated with CK2α antibody as detailed in the Materials and methods section. The immunocomplexes were then subjected to Western-blot analysis with either anti-CK2α (A) or anti-phosphotyrosine (anti-PTyr) antibodies (B) as described in the Materials and methods section. The position of recombinant CK2α tyrosine-phosphorylated by incubation with c-Fgr and ATP-Mn²⁺ is shown in the lane 'Ctrl'.

CK2α	Human	KVLGTEDLYDYIDK
CK2α'	Human	KVLGTEDLYGYLKK
CK2α	Rabbit	KVLGTEDLYDYIDK
CK2α	Mouse	KVLGTEDLYDYIDK
CK2α'	Mouse	KVLGTEDLYGYLKK
CK2α	Bovine	KVLGTEDLYGYLKK
CK2α	Chicken	KVLGTEDLYDYIDK
CK2α'	Chicken	KVLGTEDLYGYLKK
CK2α'	<i>Xenopus laevis</i>	KVLGTEDLYDYIDK
CK2α	Drosophila	KVLGTEDLYAYLDK
CK2α	<i>Caenorhabditis elegans</i>	KVLGTEDLYEYIAR
CK2α	<i>Arabidopsis thaliana</i>	KVLGTEDLYNAYLNK
CK2α'	<i>A. thaliana</i>	KVLGTEDLYNAYLNK
CK2α	Maize	KVLGTEDLYNAYLNK
CK2α	Rice	KVLGTEDLYNAYLNK
CK2α	<i>Neurospora crassa</i>	KVLGTEDLYFDYLDK
CK2α	<i>Yarrowia lipolytica</i>	RVLGTEDLYWYLDK
CK2α	<i>Schizosaccharomyces pombe</i>	KVLGTEDLYFAYVQK
CK2α	<i>Saccharomyces cerevisiae</i>	KVLGTEDLYFEKYLK
CK2α'	<i>S. cerevisiae</i>	TVLGTEDLYGYLKK
CK2α	<i>Encephalitozoon cuniculi</i>	RILGYTHLKKYVRK

Figure 8 Tyr²⁵⁵ preceded by the acidic-hydrophobic motif is conserved in animals, but not in yeast and plant CK2 catalytic subunits

The sequence containing Tyr²⁵⁵ in human CK2α has been aligned with the homologous sequences of CK2 catalytic subunits from other organisms, as indicated, using the CLUSTAL X program (<http://www-igbmc.u-strasbg.fr/BioInfo/>).

one (Tyr¹⁸²) is the main target of autocatalytic CK2 tyrosine phosphorylation as well as of two tyrosine residues at the end of the p + 1 loop, of which one (Tyr²⁰⁹) is orientated towards the active site.

HPLC and MS of trypsin-digested CK2α phosphorylated by c-Fgr have unambiguously identified Tyr²⁵⁵ as one of the phosphorylation sites. Tyr²⁵⁵ belongs to helix-H and its side chain is exposed towards the solvent [34] and therefore accessible to heterologous kinases. It also displays the optimal consensus sequence of Src phosphorylation sites [37] for having a hydrophobic residue at the crucial n-1 position (Leu²⁵⁴) and two acidic residues upstream from this. Consequently, a synthetic peptide reproducing the 248–268 sequence of human CK2α is phosphorylated readily by c-Fgr and Lyn exclusively at Tyr²⁵⁵, but not at Tyr²⁵⁷ or at Tyr²⁶¹, both of which lack the hydrophobic determinant at position n-1. Interestingly, both Tyr²⁵⁵ and the consensus triplet E/D-E/D-L, adjacent to its N-terminal side, are conserved in animal CK2 catalytic subunits from *Caenorhabditis elegans* to human, whereas in plants and in yeast, where *sensu stricto* tyrosine kinases are not present, Tyr²⁵⁵ is replaced by non-phosphorylatable residues (see Figure 8).

The phosphorylation of Tyr²⁵⁵ alone cannot account for the entire tyrosine phosphorylation of CK2 α whose stoichiometry approaches 2. Indeed, the HPLC profile of the tryptic digest of ³²P-Tyr-phosphorylated CK2 α would indicate that, besides Tyr²⁵⁵ (accounting for the most retarded radioactive peak of Figure 3A), additional residues are also phosphorylated, albeit to a lesser degree. In fact, a Y255F mutant of human CK2 α as well as maize CK2 α , which lacks Tyr²⁵⁵ (replaced by asparagine), are both phosphorylated by Lyn and c-Fgr, albeit to a lesser extent than the human catalytic subunits (see Figures 1 and 2B). More interestingly, the residual phosphorylation of the Y255F mutant and maize CK2 α , is not correlated with any significant increase in catalytic activity (Figure 5), consistent with the view that Tyr²⁵⁵ is indeed responsible for the increase in catalytic activity promoted by tyrosine phosphorylation of human CK2 α .

The correlation between tyrosine phosphorylation of CK2 and increased CK2 activity contrasts with previous report [18] that suggested that tyrosine phosphorylation of CK2 by Abl was inhibitory. The reasons for this discrepancy are not clear at the present time. The assay of CK2 was performed in both cases using very similar peptide substrates and incubation media were almost identical. The most obvious difference in experimental conditions is the nature of the tyrosine kinases used (i.e. Abl instead of Src), which may result in the phosphorylation of different residues. It should also be noted that, in that work, the inhibitory effect of Abl-catalysed phosphorylation was inferred indirectly from an increase in CK2 activity that resulted from treating tyrosine-phosphorylated CK2 α with a protein tyrosine phosphatase.

Is Tyr²⁵⁵ also phosphorylated *in vivo*? Although a definitive answer is not available currently, we have obtained evidence that endogenous CK2 α becomes tyrosine-phosphorylated on treatment of Jurkat cells with pervanadate, a potent inhibitor of protein tyrosine phosphatases. However, the very low level of tyrosine phosphorylation observed *in vivo* has hindered our attempts to assess whether this phosphorylation is attributable to heterologous phosphorylation by Src kinases or whether it was an autocatalytic event affecting Tyr¹⁸². By comparing the Western blots with anti-CK2 α and anti-phosphotyrosine antibodies (Figure 7) it appears that only a minority of CK2 molecules are tyrosine-phosphorylated in Jurkat cells. These are characterized by an up-shift, whereas the bulk of CK2 α , recognized by anti-CK2 α antibody but not by anti-phosphotyrosine antibody was not up-shifted.

To the best of our knowledge, this is the first direct evidence that endogenous CK2 α is tyrosine-phosphorylated in intact cells. Whereas the occurrence of CK2 phosphorylation at tyrosine residues has been reported in [18], it is noteworthy that those experiments were performed by transfecting an excess of recombinant CK2 α into NIH-3T3 cells. Moreover, a direct examination of the tyrosine phosphorylation of endogenous CK2 was not reported.

Bearing in mind that the CK2 holoenzyme is less prone to tyrosine phosphorylation *in vitro* than the isolated catalytic subunits, one could speculate that, within cells, the pool of catalytic subunits that is not incorporated into the holoenzyme is especially susceptible to this kind of modification. The precise existence of these free catalytic subunits in non-transfected cells has not been unambiguously proven, although it would be consistent with the structure of the holoenzyme [38] and a number of previous observations (see e.g. [39,40]). Moreover, very little is known about the kinetics of assembly of CK2 subunits within intact cells, a process that could be affected deeply by promiscuous interactions with other cellular proteins [41]. Our results are consistent with a scenario whereby, on activation of Src family kinases, a pool of CK2, represented

typically by catalytic subunits not associated with the β -subunits, becomes tyrosine-phosphorylated with a resultant increase in activity towards a subset of specific targets. These targets may include proteins such as calmodulin, whose phosphorylation is prevented by association with the β -subunits [3]. However, it should also be noted that stimulation of catalytic activity by tyrosine phosphorylation persists after association with the β -subunits, as shown by enhanced β -subunit phosphorylation within tyrosine-phosphorylated holoenzyme when compared with non-phosphorylated holoenzyme (see Figure 6). It is possible therefore that stimulation by tyrosine phosphorylation represents a regulatory mechanism that is not restricted to the free catalytic subunits alone but also affects the CK2 holoenzyme.

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REFERENCES

- Pinna, L. A. (1990) Casein kinase 2: an 'eminence grise' in cellular regulation? *Biochim. Biophys. Acta* **1054**, 267–284
- Allende, J. E. and Allende, C. C. (1995) Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *FASEB J.* **9**, 313–323
- Pinna, L. A. and Meggio, F. (1997) Protein kinase CK2 ('casein kinase-2') and its implication in cell division and proliferation. *Prog. Cell Cycle Res.* **3**, 77–97
- Guerra, B. and Issinger, O.-G. (1999) Protein kinase CK2 and its role in cellular proliferation, development and pathology. *Electrophoresis* **20**, 391–408
- Meggio, F., Marin, O. and Pinna, L. A. (1994) Substrate specificity of protein kinase CK2. *Cell Mol. Biol. Res.* **40**, 401–409
- Wilson, L. K., Dhillon, N., Thorne, J. and Martin, G. S. (1997) Casein kinase II catalyzes tyrosine phosphorylation of the yeast nucleolar immunophilin Fpr3. *J. Biol. Chem.* **272**, 12961–12967
- Tawfic, S., Yu, S., Faust, R., Davis, A. and Ahmed, K. (2001) Protein kinase CK2 signal in neoplasia. *Histol. Histopathol.* **16**, 573–582
- Seldin, D. C. and Leder, P. (1995) Casein kinase II α transgene-induced murine lymphoma: relation to theileriosis in cattle. *Science* **267**, 894–897
- Kelliher, M. A., Seldin, D. C. and Leder, P. (1996) Tal 1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase II α . *EMBO J.* **15**, 5160–5166
- Landesman-Bollag, E., Channavajhala, P. L., Cardiff, R. D. and Seldin, C. D. (1998) p35 deficiency and misexpression of protein kinase CK2 α collaborate in the development of thymic lymphomas in mice. *Oncogene* **23**, 2965–2974
- Orlandini, M., Semplici, F., Ferruzzi, R., Meggio, F., Pinna, L. A. and Oliviero, S. (1998) Protein kinase CK2 α' is induced by serum as a delayed early gene and cooperates with Hs-ras in fibroblast transformation. *J. Biol. Chem.* **273**, 21292–21297
- Li, D., Dobrowolska, G., Aicher, L. D., Chen, M., Wright, J. H., Drueckes, P., Dunphy, E. L., Munar, E. S. and Krebs, E. G. (1999) Expression of the casein kinase 2 subunits in Chinese hamster ovary and 3T3 L1 cells provides information on the role of the enzyme in cell proliferation and the cell cycle. *J. Biol. Chem.* **274**, 32988–32996
- 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
- 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
- Marin, O., Meggio, F., Sarno, S., Cesaro, L., Pagano, M. A. and Pinna, L. A. (1999) Tyrosine versus serine/threonine phosphorylation by protein kinase casein kinase-2. *J. Biol. Chem.* **274**, 29260–29265
- Donella-Deana, A., Cesaro, L., Sarno, S., Brunati, A. M., Ruzzene, M. and Pinna, L. A. (2001) Autocatalytic tyrosine phosphorylation of protein kinase CK2 α and α' subunits: implication of Tyr-182. *Biochem. J.* **357**, 563–567
- Johnson, L. N., Noble, M. E. M. and Owen, D. J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell (Cambridge, Mass.)* **85**, 149–158

- 18 Heriche, J.-K. and Chambaz, E. M. (1998) Protein kinase CK2 α is a target for the Abl and Bcr-Abl tyrosine kinases. *Oncogene* **17**, 13–18
- 19 Szyzka, R., Grankowski, N., Felczak, K. and Shugar, D. (1995) Halogenated benzimidazoles and benzotriazoles as selective inhibitors of protein kinases CK I and CK II from *Saccharomyces cerevisiae* and other sources. *Biochem. Biophys. Res. Commun.* **208**, 418–424
- 20 Sarno, S., Vaglio, P., Meggio, F., Issinger, O. G. and Pinna, L. A. (1996) Protein kinase CK2 mutants defective in substrate recognition. Purification and kinetic analysis. *J. Biol. Chem.* **271**, 10595–10601
- 21 Sarno, S., Marin, O., Ghisellini, P., Meggio, F. and Pinna, L. A. (1998) Biochemical evidence that the N-terminal segments of the α -subunit and the β -subunit play interchangeable roles in the activation of protein kinase CK2. *FEBS Lett.* **441**, 29–33
- 22 Sarno, S., Marin, O., Boschetti, M., Pagano, M. A., Meggio, F. and Pinna, L. A. (2000) Cooperative modulation of protein kinase CK2 by separate domains of its regulatory β -subunit. *Biochemistry* **40**, 12324–12329
- 23 Sarno, S., Ghisellini, P., Cesaro, L., Battistutta, R. and Pinna, L. A. (2001) Generation of mutants of CK2 α which are dependent on β -subunit for catalytic activity. *Mol. Cell. Biochem.* **227**, 13–19
- 24 Sarno, S., Ghisellini, P. and Pinna, L. A. (2002) Unique activation mechanism of protein kinase CK2. The N-terminal segment is essential for constitutive activity of the catalytic subunit but not of the holoenzyme. *J. Biol. Chem.* **277**, 22509–22514
- 25 Donella-Deana, A., James, P., Staudenmann, W., Cesaro, L., Marin, O., Brunati, A. M., Ruzzene, M. and Pinna, L. A. (1996) Isolation from spleen of a 57 kDa protein substrate of the tyrosine kinase Lyn: identification as a protein disulphide isomerase related protein and localisation of the phosphorylation sites. *Eur. J. Biochem.* **235**, 18–25
- 26 Brunati, A. M., James, P., Donella-Deana, A., Matoskova, B., Robbins, K. C. and Pinna, L. A. (1993) Isolation and identification of two protooncogene products related to *c-fgr* and *c-fyn* in a protein tyrosine fraction of rat spleen (TPK-III). *Eur. J. Biochem.* **216**, 323–327
- 27 Brunati, A. M., James, P., Guerra, B., Ruzzene, M., Donella-Deana, A. and Pinna, L. A. (1996) The spleen protein-tyrosine kinase TPK-IIb is highly similar to the catalytic domain of p72^{src}. *Eur. J. Biochem.* **240**, 400–407
- 28 Brunati, A. M., Guillaume, A., Marin, O., Donella-Deana, A., Cesaro, L., Bougeret, C., Fagard, R., Benarous, R., Fischer, S. and Pinna, L. A. (1992) Spleen protein tyrosine kinases TPK-IIb and CSK are distinct enzymes. *FEBS Lett.* **313**, 291–294
- 29 Meggio, F., Donella, A. and Pinna, L. A. (1976) A new procedure for the measurement of the protein-kinase-catalyzed incorporation of the γ -³²P-phosphoryl group of ATP into phospho-proteins and phosphopeptides. *Anal. Biochem.* **71**, 583–587
- 30 Glass, D. B., Masaracchia, R. A., Feramisco, J. R. and Kemp, B. E. (1978) Isolation of phosphorylated peptides and proteins on ion exchange papers. *Anal. Biochem.* **87**, 566–575
- 31 Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858
- 32 Larsen, M. R., Sorensen, G. L., Fey, S. J., Larsen, P. M. and Roepstorff, P. (2001) Phospho-proteomics: evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics* **1**, 223–238
- 33 Sarno, S., Reddy, H., Meggio, F., Ruzzene, M., Davis, S. P., Donella-Deana, A., Shugar, D. and Pinna, L. A. (2001) Selectivity of 4,5,6,7-tetrabromo-benzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). *FEBS Lett.* **496**, 44–48
- 34 Niefind, K., Guerra, B., Pinna, L. A., Issinger, O. G. and Schomburg, D. (1998) Crystal structure of the catalytic subunit of protein kinase CK2 from *Zea mays* at 2.1 Å resolution. *EMBO J.* **17**, 2451–2462
- 35 Brunati, A. M., Bordi, L., Clari, G., James, P., Quadroni, M., Baritono, E., Pinna, L. A. and Donella-Deana, A. (2000) Sequential phosphorylation of protein band 3 by Syk and Lyn tyrosine kinases in intact human erythrocytes. Identification of primary and secondary phosphorylation sites. *Blood* **96**, 1550–1557
- 36 Gutwein, P., Oleszewski, M., Mechttersheimer, S., Agmen-Levin, N., Krauss, K. and Altevogt, P. (2000) Role of Src kinases in the ADAM-mediated release of L1 adhesion molecule from human tumor cells. *J. Biol. Chem.* **275**, 15490–15497
- 37 Ruzzene, M., Songyang, Z., Marin, O., Donella-Deana, A., Brunati, A. M., Guerra, B., Agostinis, P., Cantley, L. C. and Pinna, L. A. (1997) Sequence specificity of C-terminal Src kinase (CSK): a comparison with Src-related kinases c-Fgr and Lyn. *Eur. J. Biochem.* **246**, 433–439
- 38 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
- 39 Stigare, J., Buddelmeijer, N., Pigon, A. and Egyhazy, E. (1993) A majority of casein kinase II α subunit is tightly bound to intranuclear components but not to the β subunit. *Mol. Cell. Biochem.* **129**, 77–85
- 40 Lebrin, F., Chambaz, E. M. and Bianchini, L. (2001) A role for protein kinase CK2 in cell proliferation: evidence using a kinase-inactive mutant of CK2 catalytic subunit α . *Oncogene* **20**, 2010–2022
- 41 Allende, C. C. and Allende, J. E. (1998) Promiscuous subunit interactions: a possible mechanism for the regulation of protein kinase CK2. *J. Cell. Biochem.* **30–31** (Suppl.), 129–136

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