Autocatalytic tyrosine-phosphorylation of protein kinase CK2 α and α' subunits: implication of Tyr¹⁸²

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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 mechanism of modulation is still obscure. Here we show that CK2 α/α' subunits undergo intermolecular (*trans*) tyrosine-autophosphorylation, which is dependent on intrinsic catalytic activity and is suppressed by the individual mutation of Tyr¹⁸², a crucial residue of the activation loop, to phenylalanine. At variance with serine-autophosphorylation,

tyrosine-autophosphorylation of CK2 α is reversed by ADP and GDP and is counteracted by the β -subunit and by a peptide reproducing the activation loop of CK2 α/α' (amino acids 175–201). These results disclose new perspectives about the mode of regulation of CK2 catalytic subunits.

Key words: autophosphorylation, 'casein kinase 2', phosphotyrosine, regulation by phosphorylation.

INTRODUCTION

Protein kinase CK2 (an acronym derived from the misnomer 'casein kinase-2') is an essential ubiquitous enzyme whose specificity is determined by multiple acidic residues mostly located downstream from the phosphorylatable amino acids [1,2]. The still increasing list of CK2 substrates includes some 200 proteins, many of which play important roles in a variety of cellular functions, with special reference to signal transduction, gene expression and metabolic regulation (see [1,3–5] for reviews). Such a pleiotropic role apparently reflects a high constitutive activity, which, in turn, is suspected to underlie the pathogenic potential of CK2 in oncogenesis and viral infections [5]. The CK2 holoenzyme is composed of two catalytic (α and/or α') and two β -subunits to form stable heterotetramers. In contrast with other protein kinases sharing a similar hetero-oligomeric structure (e.g. protein kinase A and cyclin-dependent kinases), however, both CK2 holoenzyme and its isolated catalytic subunits have constitutive activity when tested with specific peptide substrates. *In vivo*, however, the situation might be different since the phosphorylation of some CK2 protein targets, exemplified by calmodulin, is inhibited by the β -subunit, whereas other protein substrates rely on the holoenzyme for phosphorylation [3,6,7]. This suggests the possibility that a pool of CK2 catalytic subunits is present in the cell, where they play a role distinct from that of the CK2 holoenzyme. In particular, high constitutive activity is conferred to CK2 catalytic subunits by unique interactions between its N-terminal segment and elements of the catalytic core, in particular the activation loop [8,9]. In the majority of protein kinases this loop includes one or two phosphoacceptor residues whose phosphorylation, either through an autocatalytic event or by an heterologous kinase, is required for activation [10]. Such a regulatory mechanism has never been reported for CK2, whose activation loop does not include either serine or threonine residues. It includes two tyrosine residues, Tyr¹⁸² and Tyr¹⁸⁸, whose phosphorylation by protein tyrosine kinases has never been shown. CK2 holoenzyme undergoes autophosphorylation at two serine residues (Ser²/Ser³) close to the N-terminus of its β -subunit and, in the presence of polybasic peptides, at serine/threonine residues of the catalytic subunit,

which have not been identified [11]. In contrast, the isolated catalytic subunits are devoid of serine/threonine autophosphorylation activity. Recently, evidence has accumulated that, although CK2 is a *bona fide* serine/threonine kinase, it also displays tyrosine kinase activity under special circumstances [12–14]. These observations suggest the possibility that autocatalytic phosphorylation of tyrosine residues, especially those implicated in the interaction between the activation loop and the N-terminal segment, might represent a specific regulatory mechanism for CK2 catalytic subunits. The results presented provide new arguments supporting such a hypothesis.

MATERIALS AND METHODS

Materials and enzymes

[γ -³²P]ATP was purchased from Amersham Pharmacia Biotech (San Francisco, CA, U.S.A.). The tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine ('PP2') and a protease-inhibitor cocktail was obtained from Calbiochem (Darmstadt, Germany) and Boehringer (Mannheim, Germany) respectively. Other reagents were purchased from Sigma (Poole, Dorset, U.K.). 4,5,6,7-Tetrabromobenzo-2-azabenzimidazole (4Br-2-azaBz) was kindly provided by Dr David Shugar (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) [15]. Anti-phosphotyrosine was from ICN Biotechnology (Irvine, CA, U.S.A.). Synthetic peptides were synthesized as detailed elsewhere [14,16–19].

Wild-type CK2 α , CK2 α mutants, CK2 α' and CK2 β were expressed in *Escherichia coli* as described elsewhere [19,20].

Tyrosine-autophosphorylation of CK2 α

CK2 α , CK2 α' and CK2 α mutants (300 nM) were autophosphorylated in 30 μ l of incubation medium containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂ (basal medium) and 0.1 mM ATP. After 20 min of incubation, the reactions were stopped and the samples were subjected to SDS/PAGE. Proteins were transferred to nitrocellulose membranes and immunostained with anti-phosphotyrosine antibodies. Filters were developed

Abbreviations used: 4Br-2-azaBz, 4,5,6,7-tetrabromobenzo-2-azabenzimidazole; CK2, protein kinase CK2.

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using an enhanced chemiluminescence detection system (ECL[®]; Amersham Pharmacia Biotech). Densitometric analysis of the CK2 α bands was performed using an ImageMaster VDS instrument (Amersham Pharmacia Biotech).

Autophosphorylation of reconstituted CK2 holoenzyme

Autophosphorylation of CK2 holoenzyme was performed in basal medium containing 50 μ M [γ -³²P]ATP and equimolar amounts of CK2 α and CK2 β (150 nM). After 15 min of incubation, the reactions were stopped and the samples were subjected to SDS/PAGE. The extent of protein ³²P phosphorylation was evaluated by analysis on a Packard Imager.

CK2 α activity towards a peptide substrate

The standard activity of CK2 α and CK2 α mutants was tested in basal medium containing 50 μ M [γ -³²P]ATP, using the peptide RRRADSDDDD (30 μ M) as substrate. The reactions were terminated after 5 min of incubation by spotting 25 μ l of the mixture on to P81 phosphocellulose papers [22].

RESULTS

Recombinant human CK2 α/α' subunits, expressed in *E. coli* and purified to near homogeneity, readily undergo tyrosine-autophosphorylation on incubation with ATP and Mg²⁺, as judged from immunostaining with anti-phosphotyrosine antibodies (Figure 1, lane 1). Similar results were obtained by phosphoradiolabelling with [³²P]ATP, followed by alkali treatment to minimize serine/threonine phosphorylation (results not shown). The autocatalytic mechanism of such a tyrosine-autophosphorylation process was confirmed by the ability of the specific CK2 co-substrate, GTP, to replace ATP as phosphate donor (Figure 1, lane 9), by the failure of a catalytically inactive

Figure 1 Tyrosine-autophosphorylation of CK2 α/α' subunits: influence of various compounds

Anti-phosphotyrosine immunostaining of tyrosine-autophosphorylated CK2 α (lane 1), CK2 α' (CK2 β ; lane 2) and CK2 α (Δ 2-12) (lane 3). In lane 4, CK2 α (300 nM) and CK2 α (Δ 2-12) (500 nM) were incubated together. In lanes 5-8, tyrosine-autophosphorylation of CK2 α was performed in the presence of the following compounds: heparin (1 μ g/ml), emodin (5 μ M), 4Br-2-azaBz (5 μ M) and PP2 (5 μ M) respectively. In lane 9, the co-substrate ATP was replaced by GTP as phosphate donor. Tyrosine-autophosphorylation of CK2 α/α' subunits was performed and quantified as described in the Materials and methods section. The immunostained band slightly more mobile than CK2 α is generated by limited proteolysis of it, as judged by immunoreaction with anti-CK2 α antibodies. The Figure is representative of five different experiments.

Figure 2 Effect of increasing enzyme concentration (A) and medium viscosity (B) on CK2 α tyrosine-autophosphorylation

Experimental conditions are detailed in the Materials and methods section. Reported values represent the means for four separate experiments, with the S.E.M. values indicated by vertical bars.

mutant, CK2 α (Δ 2-12) (CK2 α with residues 2-12 deleted), to undergo tyrosine-autophosphorylation (Figure 1, lane 3) and by the effect of specific inhibitors of CK2, such as heparin, emodin and 4Br-2-azaBz, to prevent tyrosine-autophosphorylation of wild-type CK2 α at concentrations that are also effective on catalytic activity (Figure 1, lanes 5-7). In contrast the tyrosine kinase inhibitor PP2 has no effect on CK2 α tyrosine-autophosphorylation (Figure 1, lane 8). Phosphotyrosine incorporation increases more than linearly by increasing the CK2 α concentration (Figure 2A), behaviour which, in conjunction with the inhibitory effect of increasing viscosity by addition of glycerol (Figure 2B), argues in favour of an intermolecular (*trans*), rather than intramolecular (*cis*) mode of autophosphorylation. Compelling evidence that this must be the case was provided by the behaviour of the intrinsically inactive deletion mutant, CK2 α (Δ 2-12), which is not in itself able to catalyse tyrosine-autophosphorylation, but is readily tyrosine-phosphorylated upon incubation with wild-type CK2 α (compare lanes 3 and 4 of Figure 1).

Interestingly, tyrosine-autophosphorylation is abolished on holoenzyme reconstitution by addition of equimolar concen-

Figure 3 Tyrosine-autophosphorylation of CK2 α is inhibited by β -subunit

CK2 α was autophosphorylated as described in the Materials and methods section, in the absence or presence of increasing concentration of either the full-length CK2 β or the peptides CK2 β (170–215) and CK2 β (181–203). Tyrosine-autophosphorylation is expressed relative to that of CK2 α alone. Reported values represent the means for five separate experiments, with the S.E.M. values indicated by the vertical bars.

tration of the β -subunit to CK2 α (Figure 3). The addition of synthetic fragments of the β -subunit, which are known to interact with the catalytic subunit, is also detrimental to tyrosine-autophosphorylation, although the overall catalytic activity of CK2 α is actually enhanced by both the β -subunit and these fragments [18]. The most likely interpretation is that the β -subunit, either directly or by inducing conformational changes, masks the tyrosine residue(s) susceptible to phosphorylation.

A notable feature of CK2 α tyrosine-autophosphorylation is its reversal on addition of ADP (Figure 4A). In contrast, owing to the difference in energy between the γ -phosphate bond of ATP and the phosphate bond of phosphopeptides, the phosphotransferase reactions catalysed by protein kinases, either serine/threonine- or tyrosine-specific, are generally not readily reversible. For the sake of comparison, CK2 holoenzyme autophosphorylated at the serine residues of its β -subunits was also incubated with ADP under comparable conditions and, as shown in Figure 4(B), this treatment did not result in any appreciable loss of radioactivity from the protein. It has to be concluded, therefore, that the phosphotyrosine residues of CK2 α are unusually reactive, possibly because they are inside or close to the active site. This conclusion is corroborated by the observation that GDP can replace ADP as phosphate acceptor (Figure 4), as would be expected when one considers the unusual ability of CK2 to use GTP as phosphate donor. High reactivity of CK2 α phosphotyrosine may account for the failure to find detectable amounts of phosphotyrosine in CK2 α recombinantly expressed and purified from bacteria. It may also explain the relatively low level of CK2 α tyrosine-autophosphorylation, which, under our conditions, is of relatively low stoichiometry (0.05–0.1 mol of phosphotyrosine/mol of protein).

The unique features of CK2 α tyrosine-autophosphorylation are further highlighted by the inhibitory effect of a synthetic peptide, CK2 α (175–201), encompassing the whole activation loop; this peptide is not phosphorylated by CK2 α and does not affect either the catalytic activity towards peptide substrates or serine-autophosphorylation of the β -subunit (results not shown). Nevertheless it potently inhibits tyrosine-autophosphorylation with an IC_{50} around 2.5 μ M (see Figure 5). This value indicates

Figure 4 Tyrosine-autophosphorylation of CK2 α is reversed in the presence of ADP and GDP

(A) Anti-phosphotyrosine immunostaining of CK2 α autophosphorylated for 20 min as in Figure 1 and then incubated for 20 min either in the absence (lane 1) or in the presence of the indicated concentrations of ADP (lanes 2 and 3) or GDP (lane 4). In (B) CK2 holoenzyme, reconstituted by combining equimolar amounts of CK2 α and CK2 β , was subjected to autophosphorylation in the presence of [γ - 32 P]ATP for 15 min and then incubated for 20 min either in the absence (lane 5) or in the presence of the indicated concentrations of ADP (lanes 6 and 7). The autoradiographies of the dried gels are shown. Radioactivity incorporated in (B) is accounted for entirely by phosphoserine/phosphothreonine, as judged by its disappearance after alkali treatment of the gel. Results are representative of three different experiments.

Figure 5 Effect of synthetic peptides on CK2 α tyrosine-autophosphorylation

Tyrosine-autophosphorylation of CK2 α was evaluated either in the absence (100%) or presence of increasing concentrations of the indicated peptides. Data are means for three separate experiments. S.E.M. values were always less than 16%.

an inhibitory efficiency higher than that of the best known CK2 tyrosyl-peptide substrate, derived from the phosphoacceptor site of immunophilin Fpr3, DEDADIYDEEDYDL. This, in turn, is much more effective as an inhibitor of tyrosine-autophosphorylation than the optimal seryl-peptide substrate RRRADDSDDDDD (IC_{50} values 11 μ M versus 40 μ M), despite its K_m value is huge as compared with that of the seryl peptide (384 μ M versus 20 μ M) [13]. We conclude, therefore, that inhibition of tyrosine-autophosphorylation by the activation loop (175–201) peptide is not merely a consequence of substrate competition, but reflects specific interactions relevant only to tyrosine-autophosphorylation.

Since the activation loop of CK2 includes two tyrosine residues (Tyr¹⁸² and Tyr¹⁸⁸), one of which makes crucial interactions with

Figure 6 Mutation of Tyr¹⁸² to phenylalanine suppresses tyrosine-autophosphorylation of CK2 α .

Tyrosine residues implicated in the interaction between the activation loop (light grey) and the N-terminal segment (dark grey) are shown. The Figure is drawn from the crystal structure of maize CK2 α [8] using Raster3D and MOLSCRIPT programs [33,34]. The inset shows the anti-phospho-Tyr immunostaining of tyrosine-autophosphorylated wild-type CK2 α and the indicated mutants. The catalytic activity of the mutants, tested toward the peptide RRRADDSDDDDD and expressed relative to wild-type CK2 α (= 100) is indicated in parentheses.

the activatory N-terminal segment, and, in turn, a tyrosine residue of the N-terminal segment (Tyr²⁶) hydrogen bonds with Glu¹⁸⁰ in the activation loop (see Figure 6) these three tyrosine residues were individually mutated to phenylalanine to assess their possible implication in the autophosphorylation event. All these mutants are catalytically active, albeit less than wild-type CK2 α . As shown in the inset of Figure 6, however, the mutant Y182F totally lost its ability to undergo tyrosine-autophosphorylation. These data strongly suggest that Tyr¹⁸² is the main, if not the only, site of tyrosine-autophosphorylation in wild-type CK2 α . However, indirect effects cannot be excluded, and they could account for the inability of the mutant Y182F to autophosphorylate even if the target tyrosine residue is present in its sequence.

DISCUSSION

The data presented provide evidence that, in the presence of ATP (or GTP) and Mg²⁺, both catalytic subunits of CK2, α and α' , undergo appreciable autophosphorylation at tyrosine residue(s), one of which is, most likely, Tyr¹⁸², located in the activation loop. The autocatalytic occurrence of CK2 α/α' tyrosine-phosphorylation is consistent with all the available data; in particular, it is susceptible to competitive inhibition by the specific peptide substrates RRRADDSDDDDD and DEDADIYDEEDYDL and it is strictly dependent on intrinsic catalytic activity, being reduced or abolished by specific CK2 inhibitors and by a mutation that suppresses CK2 α enzymic activity. A *trans* (intermolecular) rather than a *cis* (intramolecular) autocatalytic mechanism appears to underlie CK2 α tyrosine-phosphorylation, since its rate increases more than linearly by increasing CK2 α concentration and decreases to

negligible values if the viscosity of the incubation medium is increased with glycerol. Consequently, an intrinsically inactive mutant of CK2 α that is not itself able to undergo tyrosine-autophosphorylation, namely CK2 $\alpha(\Delta 2-12)$, becomes readily tyrosine-phosphorylated upon incubation with wild-type CK2 α .

Although CK2 α/α' tyrosine-autophosphorylation is an intermolecular event depending on catalytic activity, it appears to be unique in several respects. First, it is prevented by association with the β -subunit, which is conversely required for serine/threonine-autophosphorylation of CK2 holoenzyme either in the absence or presence of polybasic peptides and generally increases catalytic activity toward most, albeit not all, CK2 targets. Secondly, tyrosine-autophosphorylation is readily reversed in the presence of an excess of the phosphoacceptor co-substrate ADP, whereas serine-autophosphorylation of CK2 holoenzyme is not. Thirdly, tyrosine-autophosphorylation is efficiently and specifically inhibited by micromolar concentration of the peptide CK2 $\alpha(175-201)$ reproducing the activation loop of CK2 α , although this peptide has no effect on catalytic activity (tested with the specific peptide substrate) nor on serine-autophosphorylation of the holoenzyme (results not shown). What makes inhibition of tyrosine-autophosphorylation by CK2 $\alpha(175-201)$ intriguing is, on the one hand the inhibitory potency of the peptide, by far superior to that of the optimal CK2 peptide substrate RRRADDSDDDDD, and, on the other hand, its failure to undergo phosphorylation by CK2 at any of its tyrosine residues. In a way this latter outcome did not come as a surprise, since none of the tyrosine residues in the CK2 $\alpha(175-201)$ peptide displays the consensus for tyrosyl-peptide phosphorylation by CK2 [13]. This, however, also applies to all the tyrosine residues of CK2 α . Taken collectively, these data would indicate that inhibition of tyrosine-autophosphorylation by the CK2 $\alpha(175-201)$ peptide cannot be explained by substrate competition alone and must involve specific interactions that do not affect the regular catalytic activity of the enzyme. On the other hand, the identification of one of the tyrosine residues included in the CK2 $\alpha(175-201)$ peptide, Tyr¹⁸², a strong candidate to be the main, if not the only, tyrosine residue modified by autophosphorylation of full-length CK2 α , is soundly grounded on the observation that its mutation to phenylalanine is sufficient to fully abolish tyrosine-autophosphorylation, although the catalytic activity of this mutant is comparable with that of other mutants (e.g. Y26F) whose tyrosine-autophosphorylation is unaffected. It has to be assumed, therefore, that autophosphorylation of Tyr¹⁸² within the α -subunit is dictated by conformational features that are missing in the synthetic peptide spanning residues 175–201. This region corresponds to the so-called 'activation loop', whose phosphorylation in most protein kinases is a prerequisite for attainment of a fully active conformation [10]. In this respect, however, CK2 represents an exception, since its active conformation does not rely on previous phosphorylation of its activation loop but is provided by extensive interaction between the activation loop and the N-terminal segment [8,9]. The sequence homologue of the residue whose phosphorylation triggers the activation of other kinases (protein kinase A Thr¹⁹⁷) in CK2 α is Asn¹⁸⁹, a residue quite a long way away from Tyr¹⁸². The side chain of this latter, moreover, is oriented in a direction opposite to the catalytic site, being engaged with the N-terminal tail, through a hydrogen bond with the backbone nitrogen atom of Ala⁹ (see Figure 6). Incidentally, the relevance of this interaction to active conformation is confirmed by the reduced catalytic activity of the Y182F mutant (inset to Figure 6). One might expect, therefore, that phosphorylation of Tyr¹⁸² will lead to down-regulation of CK2 α/α' by weakening the overall interaction between the

activation loop and the N-terminal segment. Experimental support for this hypothesis could not be provided up until now, owing to the low stoichiometry of CK2 α / α' tyrosine-autophosphorylation and the failure to achieve a satisfactory separation of the phosphorylated from unphosphorylated form of the kinase by immunoprecipitation with anti-phosphotyrosine antibody. This may be a consequence of the instability of this tyrosine-phosphate linkage, consistent with the reversal of the autophosphorylation reaction observed in the presence of ADP (see Figure 4A).

While the physiological relevance of CK2 tyrosine autophosphorylation is a matter of conjecture, the available data are consistent with a scenario whereby the overall activity of intracellular CK2 catalytic subunits is determined by the balance between Tyr¹⁸² autophosphorylation and -dephosphorylation, the former prevailing over the latter only under conditions where CK2 α / α' concentration becomes exceedingly high (e.g. in special subcellular compartments) and the ATP/ADP ratio is also high. From our *in vitro* experiments it would appear that the majority of CK2 molecules, present in the cell as heterotetrameric holoenzyme, are refractory to this mode of regulation. This might conversely play a critical role in down-regulating pools of free CK2 catalytic subunits, whose presence in living cells is made plausible by a number of observations (see, e.g. [23–26]), and which under certain experimental conditions, such as transgenic mice [27–29] and cultured cells transfected with CK2 α / α' [30,31] is suspected to display oncogenic potential. Pertinent to this may also be the observation that tyrosine-phosphorylation of CK2 α co-transfected with p190Bcr-Abl in NIH3T3 cells correlates with a decrease in catalytic activity [32]. It will be important to assess whether, in this case also, Tyr¹⁸² is involved and/or if the same residue can be phosphorylated *in vitro* by Abl and possibly by other protein-tyrosine kinases as well.

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