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Discrimination between the activity of protein kinase CK2 holoenzyme and its catalytic subunits

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Abstract The acronym CK2 denotes a highly pleiotropic Ser/Thr protein kinase whose over-expression correlates with neoplastic growth. A vexed question about the enigmatic regulation of CK2 concerns the actual existence in living cells of the catalytic (α and/or α′) and regulatory β-subunits of CK2 not assembled into the regular heterotetrameric holoenzyme. Here we take advantage of novel reagents, namely a peptide substrate and an inhibitor which discriminate between the holoenzyme and the catalytic subunits, to show that CK2 activity in CHO cells is entirely accounted for by the holoenzyme. Transfection with individual subunits moreover does not give rise to holoenzyme formation unless the catalytic and regulatory subunits are co-transfected together, arguing against the existence of free subunits in CHO cells.

Keywords: CK2 peptide substrate; CK2 activity assay; CK2 holoenzyme

1. Introduction

Protein kinase CK2 (an acronym derived from the misnomer "casein kinase" 2) is one of the most pleiotropic members of the eukaryotic "kinome", with a list of more than 300 protein substrates already identified [1], implicated in a variety of cellular functions [2]. At variance with the great majority of Ser/Thr protein kinases, which are basophilic and/or proline directed enzymes, CK2 recognizes phosphoacceptor residues specified by multiple carboxylic and/or pre-phosphorylated amino acids, with the one at position $n + 3$ playing an especially crucial role ([1] and references therein). Such a peculiar site specificity has been exploited to generate very specific peptide substrates which not only are unaffected by basophilic and Pro-directed kinases, but also neatly discriminate CK2 activity from those of the other few acidophilic Ser/Thr kinases [3], making possible the sensitive and precise determination of CK2 activity in crude extracts and in living cells [4]. Unlike the majority of protein kinases, CK2 is not turned on in response to specific stimuli. Such a constitutive activity is suspected to underlie the oncogenic potential of CK2 whose catalytic subunits, besides being causative of transformation per se upon transfection, dramatically enhance the tumour phenotype induced by altered expression of oncogenes or tumour suppressor genes (reviewed in [5]). These data did not entirely come as a surprise considering that CK2 activity is invariably elevated in many different kinds of tumours (reviewed in [6]). These observations substantiate the raising concept that CK2 may represent a valuable target in cancer therapy (see e.g. Ref. [7]).

A pertinent question about the pathogenic potential of CK2 is whether it is promoted by abnormally high levels of CK2 holoenzyme or by an unbalanced excess of its un-assembled catalytic subunits. Indeed this is one facet of a more general issue, concerning the mode of regulation of this enigmatic kinase, whose holoenzyme displays a quaternary structure composed by two catalytic (α and/or α′) and two "regulatory" β-subunits. Although the catalytic subunits are constitutively active either alone or combined with the β-subunits, these latter deeply influence many other properties of the kinase, with special reference to specificity, association with intracellular partners and responsiveness to polybasic effectors [2,8]. It would be tempting therefore to speculate that complexed and non-complexed CK2 subunits might exist in equilibrium [2], considering that the nature and surface of the contacts between the β and α subunits make plausible the hypothesis that the holoenzyme might dissociate [9]. This led to conceptual speculations about sophisticated modes of regulation of CK2 [10,11] based on the transient nature of CK2 holoenzyme and its dynamic association–dissociation eventually giving rise to complexes in which CK2 subunits are bound to other proteins.

While the scenario proposed is appealing and, from time to time, data suggesting the presence in cells of un-combined CK2 subunits appear in the literature (e.g. [12–17]), the incontrovertible proof of concept for the existence of pools of non-assembled CK2 subunits has been frustrated by a number of shortcomings: (1) Quantitative western blot analyses of catalytic versus β CK2 subunits are not reliable due to the sharply different immunoreactivity of available antibodies, as also shown in this paper (see Fig. 2A). (2) Quantification based on molecular weight estimation is biased by the formation of supra-molecular complexes. (3) The specific peptide substrates commonly employed for determining CK2 activity are phosphorylated with similar efficiencies by either the holoenzyme or the isolated catalytic subunits. (4) Likewise inhibitors capable to neatly discriminate between free and assembled CK2 catalytic subunits were not available.

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Here we describe two novel reagents designed to overcome hindrances outlined in 3 and 4 and their successful usage to discriminate between activities of CK2 catalytic subunits and holoenzyme in the lysates of CHO cells either as such or transfected with individual CK2 subunits.

2. Methods

2.1. Peptides

The synthesis of the peptide MSGDEMIFDPTMSKKKKKKKKP was performed by solid phase using an automated peptide synthesizer (model 431-A, Applied Biosystems) and will be detailed elsewhere.

2.2. Plasmid constructs for CK2 subunits

CK2α and CK2β human cDNA were amplified by PCR from the pT7-7 vector. PCRs were performed with Pfu DNA polymerase (Promega) for 28 cycles in a programmable thermal controller. Primer sequences were: 5′-GCGGCGACCATGGCGGGACCCGTG-3′ (forward primer) and 5′-TGCCTGAGCGCCAGCGGCAGCTG-3′ (reverse primer). PCR fragments were gel purified, polyadenylated and subcloned into pGEM-T Easy vector (Promega). pGEM-T Easy vector was digested with *Eco*RI and the 1.2 kb fragment, obtained from *Eco*RI digestion, was gel purified and ligated into the corresponding restriction enzyme site of pcDNA™3.1/*myc*-His(−)A (Invitrogen) to generate the two expression constructs. Two stop codons, generated in the cloning process between the cDNA and c-*myc* and His tags, were mutated with the "QuickChange-Site Directed Mutagenesis" Kit (Stratagene) and the two used primers were 5′-TCAGCAGGCAAATCACGCGGCAATTCCACCACACTGG-3′ (forward primer) and 5′-CCAGTGTGGTGGAATTGCCGCGTGATTTGCCTGCTGAGCGC-3′ (reverse primer). Sequencing was used to verify the two final constructs.

2.3. Cell culture, transfection and immunoprecipitation

Chinese Hamster Ovary (CHO) cells were grown in Ham's F10 medium supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine, and antibiotics (penicillin, 100 U/ml, streptomycin 0.1 mg/ml) at 37 °C in a 5% CO_2 incubator. Subconfluent CHO cells grown in 6-well dishes were transfected with pcDNA™3.1/*myc*-His(−) vector expressing CK2α and/or CK2β and with pcDNA™3.1/*myc*-His(−) empty vector, as control, with GeneJammer (Statagene) according to the manufacturer's instructions. After 48 h of transfection, cells were centrifuged, washed and lysed by the addition of 60 μl of ice-cold buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.5% (v/v) Triton X-100, 2 mM dithiothreitol, protease inhibitor cocktail Complete (Roche). After 20 min incubation on ice, the lysates were centrifuged at 10 000 × *g* for 10 min, at 4 °C and proteins of soluble fractions were determined by the Bradford method. Equal amounts (5 μg) were loaded on 15% SDS/PAGE, blotted on Immobilon-P membranes (Millipore), and processed in Western blot with the indicated antibody, detected by ECL (Enhanced Chemiluminescence, Amersham Biosciences). Cells designated for IP were washed and lysed as described above. Two hundred micrograms of cell extracts were incubated with anti-CK2α overnight and immunoprecipitated by addition of protein A-Sepharose (1 h at 4 °C). The IP was washed three times, subjected to SDS/PAGE, transblotted and developed with anti-myc and subsequent anti-CK2α and anti-CK2β antibodies.

2.4. Phosphorylation assays

CK2 activity was determined as previously described [18] following the P-cellulose filters procedure. CK2 activity of cell lysate (1 μg) was tested in the same buffer using 333 μM synthetic peptides RRRADDSDDDDD and MSGDEMIFDPTMSKKKKKKKKP.

3. Results

While the β-subunit can influence the substrate specificity of CK2 by altering the phosphorylation efficiency of some protein targets in opposite directions (reviewed in [8]), its effect on peptide substrates is in general less pronounced and unidirectional, typically resulting in a modest increase in phosphorylation rate as compared to the catalytic subunits alone. Therefore commonly used CK2 peptide substrates cannot discriminate between holoenzyme and isolated catalytic subunits [19]. A unique exception is provided by a recently synthesized peptide reproducing the N-terminal segment of the eukaryotic translation initiation factor 2β (eIF2β). This protein is phosphorylated by CK2 at residue Ser2 in a fashion which is entirely relying on the CK2β-subunit [20], a property exceptionally maintained by the peptide (eIF2β1–22), whose phosphorylation is also catalyzed only by CK2 holoenzyme (Fig. 1).

We therefore decided to exploit this peptide to evaluate precisely the activity of CK2 holoenzyme in cell lysates and to gain information about the presence of free CK2 subunits in them. In particular we reasoned that, if present, these should give rise to CK2 holoenzyme upon transfection of either the CK2α or the CK2β subunits into the cells, thus reflecting in increased activity toward the (eIF2β1–22) peptide. To discriminate between endogenous and transfected pools of CK2 subunits the latter were constructed with a short Myc-His tag causing a significant up-shift upon PAGE/SDS and allowing specific immuno-detection using anti-tag antibodies.

As shown in Fig. 2A, where Western blots of cell lysates with anti-Myc antibodies is shown, transfection of either CK2α or CK2β subunits or both gave rise to the expected patterns, with transfected subunits detectable as intense bands. For sake of quantitative comparison between transfected (Myc-His tagged) and endogenous CK2 subunits the same gels were also developed with anti-α and anti-β antibodies (Fig. 2B). This highlights two points: (i) transfected Myc-His-CK2α is largely predominant over endogenous CK2α, with a 4:1 ratio calculated by densitometric analysis (not shown); (ii) The sensitivity of the anti-CK2β antibody is lower than that of the anti-α one, since with this antibody the Myc-His-CK2β band is much fainter than the Myc-His-CK2α one (Fig. 2B, lane 4) while using the same antibody (anti-Myc) their intensities were comparable (see lane 4 in Fig. 2A). Consequently endogenous CK2β is not detectable at all (lane 1, control) so that, based on this

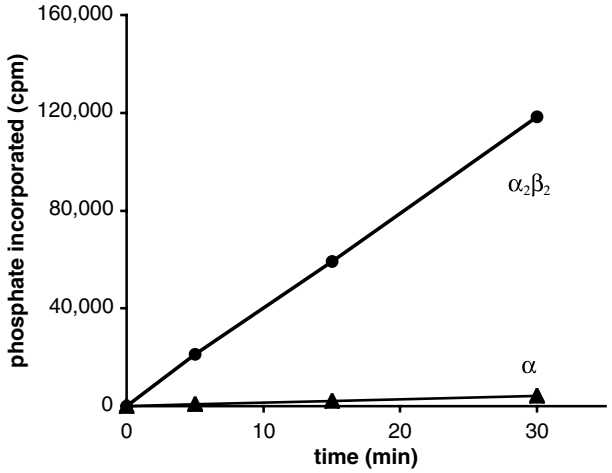

Fig. 1. The eIF2β[1–22] peptide is phosphorylated by CK2 holoenzyme but not by CK2α subunit. Phosphorylation reaction was performed as described in Section 2. The data represent the mean from three independent experiments. Similar results were obtained using the other catalytic subunit of CK2, α′.

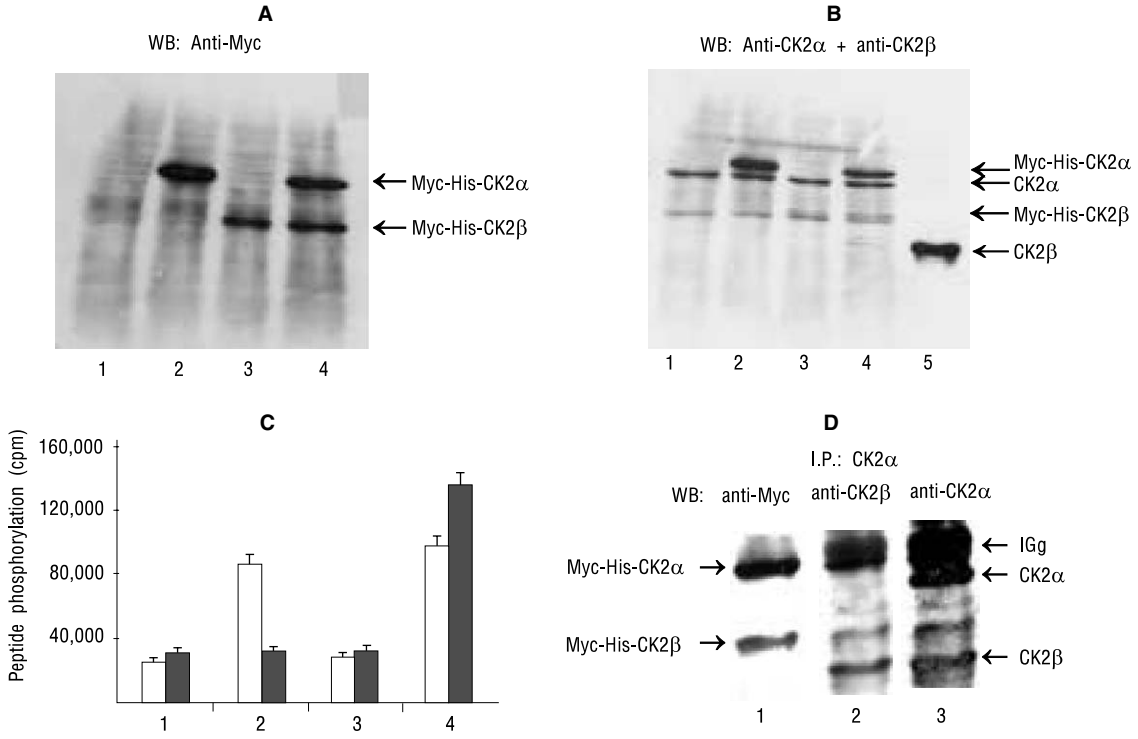

Fig. 2. Transient overexpression of CK2 subunits in CHO cells. CHO cells were transfected with control vector (lane 1), with Myc-His-tagged CK2α (lane 2), with Myc-His-tagged CK2β (lane 3), or cotrasfected with Myc-His-tagged CK2α plus Myc-His-tagged CK2β (lane 4). Cells lysates were examined by immunoblotting using monoclonal anti-Myc (A) and anti-CK2α plus CK2β antibodies (B). Recombinant β subunit is shown in lane 5. (C) CK2 activity in crude lysate of transfected cells was determined using either the peptide substrate eIF2β (filled bars) or RRRADDSDDDDD (empty bars). The mean values ± S.D. from triplicate assays are reported. (D) Cell lysates of cotransfected cells were treated with anti-CK2α antibody, immunoprecipitated with protein A sepharose, subjected to SDS/PAGE, transblotted and developed with anti-myc (lane 1), subsequently with anti-CK2β (lane 2) and anti-CK2α (lane 3) antibodies.

AB, one would get the wrong impression that in CHO cells lysates CK2 is only present with its isolated α subunit. That this is not the case is confirmed by the activity monitored with the eIF2β peptide which is not phosphorylated by the isolated catalytic subunits: such an activity is quite significant in non-transfected cells, consistent with the presence of the holoenzyme in them, and is not increased upon CK2β transfection, ruling out the presence of significant amounts of isolated CK2α expected otherwise to associate with transfected CK2β. Likewise transfection of CK2α does not give rise to any increase in activity monitored with the eIF2β peptide, whereas a fourfold increase is monitored using a canonical CK2 peptide substrate (Fig. 2C), exactly corresponding to the increase in CK2α protein evaluated by western blot (compare Fig. 2C with Fig. 2B, lane 2). This outcome supports the view that in CHO cells there is no pool of free CK2β subunits ready to associate with CK2α. The confirmation that the eIF2β peptide provides a reliable titration of CK2 holoenzyme is provided by bar 4 in Fig. 2C showing that an increase of activity roughly proportional to the amount of transfected α is indeed detectable provided that the β subunit is transfected together with the CK2α one (compare bars 2 and 4).

The formation of CK2 holoenzyme upon transfection of both the CK2α and the CK2β subunits in CHO cells was confirmed by co-immunoprecipitation experiments, as shown in Fig. 2D. Lysates of co-transfected cells were treated with anti-CK2α antibodies; the IPs were subjected to PAGE/SDS, transblotted and developed with anti-Myc antibodies: these revealed the presence also of the recombinant CK2β subunit in the immunoprecipitate (lane 1). By subsequent development of the stripe with anti CK2α and anti-CK2β antibodies the presence of wild type CK2α and CK2β subunits was also detected (lanes 2 and 3), consistent with the presence of the endogenous CK2 holoenzyme, besides the recombinant one in transfected cells. Note that CK2α subunit could be also immunodetected in the anti-Myc immunoprecipitates (not shown) consistent with the view that both native and transfected subunits are incorporated into the same molecules of heterotetrameric holoenzyme.

To validate the above conclusion by an independent pharmacological approach advantage has been taken of MNA (1,8-dihydroxy-4-nitro-antracene-9,10-dione) [18], a CK2 inhibitor whose efficacy on the holoenzyme is 10-fold higher than on CK2α. As shown in Fig. 3A CK2 holoenzyme and the isolated CK2α subunit are inhibited by MNA with IC_{50} values of 0.3 and 2.8 μM respectively. The IC_{50} value for inhibition of endogenous CK2 activity in CHO lysates is 0.35 μM, whereas comparable inhibition of CK2 activity in lysates of CK2α transfected cells requires much higher concentration of MNA (Fig. 3B). These data corroborate the view that endogenous activity is accounted for by CK2 holoenzyme, while transfected CK2α remains in its monomeric form.

4. Discussion

We have developed two biochemical tools for discriminating between the activities of protein kinase CK2 holoenzyme

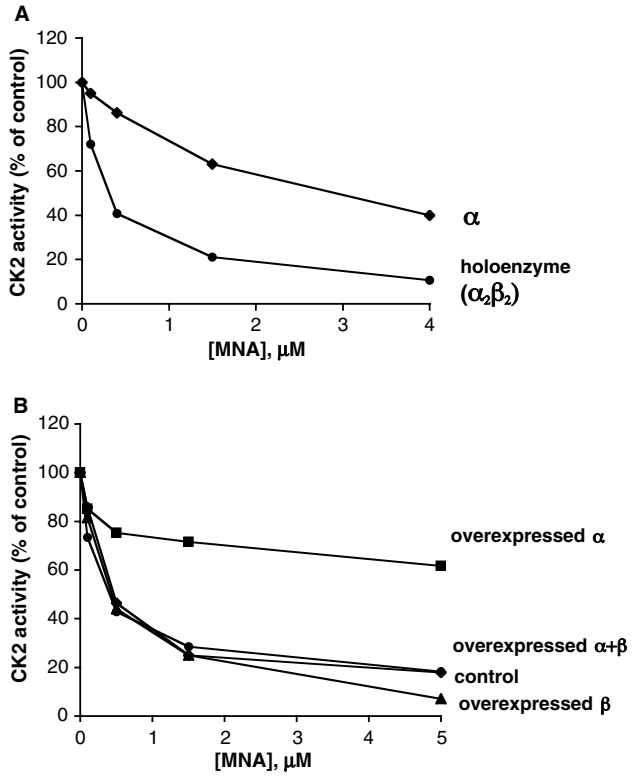

Fig. 3. Inhibition of CK2 activity by MNA. CK2 activity of the purified recombinant enzyme (A) and of CHO cell lysates (B) was assayed as described in Section 2 by using the specific peptide substrate RRRADDSDDDDD either in the absence or in the presence of increasing concentrations of MNA inhibitor. The data represent the mean of experiments run in triplicate with S.E. never exceeding 10%.

and of its isolated catalytic subunits. One is based on a unique peptide substrate (eIF2β1–22) which, unlike all the CK2 peptide substrates described so far, is phosphorylated exclusively by the holoenzyme, but not by the catalytic subunits. The other relies on an ATP site directed CK2 inhibitor (MNA) whose potency is 10-fold higher on the holoenzyme as compared to the isolated catalytic subunits. By both criteria it appears that CK2 activity in CHO cell lysates is exclusively accounted for by the holoenzyme while in the lysates of the same cells transfected with CK2α subunits the activity is predominantly due to the catalytic subunit not incorporated into holoenzyme.

These conclusions, as far as CHO cells are concerned, are grounded on the following main outcomes:

(i) The lysates of non-transfected CHO cells and cells transfected with the β subunit display comparable CK2 activities monitored either with the eIF2β peptide or with a conventional peptide which is phosphorylated by both CK2 holoenzyme and catalytic subunits.

(ii) Transfection of CHO cells with fourfold molar excess recombinant Myc-His-tagged CK2α over wild type endogenous α promotes a fourfold rise in CK2 activity monitored with the conventional peptide, without any change in activity monitored with the eIF2β peptide.

(iii) Transfection of CHO cells with both recombinant CK2α and CK2β CK2 subunits leads to a comparable increase in activity monitored with either the conventional or the eIF2β peptide.

(iv) The IC_{50} value for CK2 inhibition by MNA in CHO cell lysates is the same observed with CK2 holoenzyme, whereas the lysates of cells transfected with CK2α are much more resistant to MNA, with an IC_{50} value comparable to that observed in vitro with CK2α.

These data not only support the view that in CHO cells CK2 activity is by far predominantly, if not exclusively, accounted for by the holoenzyme, but also argue against the presence in these cells of significant pools of free catalytic and regulatory CK2 subunits, at least under the basal conditions tested. Their presence in fact would have been revealed by an increase of activity toward the eIF2β peptide upon transfection with either the CK2α or the CK2β subunits alone as a consequence of the formation of new holoenzyme molecules. Such a formation, whenever the catalytic and regulatory subunits are transfected together inside the cell, is well documented both by the increase of the activity toward the eIF2β peptide (Fig. 2C) and by the co-immunoprecipitation of the two subunits upon addition of anti-CK2α antibodies (Fig. 3A). Likewise the presence of free CK2β-subunits, sometimes reported under special conditions [12–17] in the case of CHO cells would be inconsistent with the observation that transfected CK2α-subunits accumulate in the cell as such without giving rise to any detectable amount of holoenzyme. Similar results have been obtained with N2A neuroblastoma and HEK-293 cells: in both cases CK2 activity monitored with the conventional peptide is comparable to that monitored using the eIF2β peptide, arguing against the presence of active CK2 catalytic subunits not assembled with the β subunit in these cells (not shown). This does not rule out the possibility that CK2 subunits might exist tightly bound to other proteins able to prevent the formation of the canonical holoenzyme. In this connection it would be interesting to assess if elevated CK2 activity found in a variety of tumours [6] is accounted for by holoenzyme or isolated catalytic subunits. This can now be done by analyzing the elevated CK2 activities found in tumour cells with respect to their ability to phosphorylate the eIF2β peptide and of being inhibited by MNA.

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