

Protein kinase CK2 phosphorylates and upregulates Akt/PKB

G Di Maira^{1,2}, M Salvi^{1,2}, G Arrigoni^{1,2}, O Marin^{1,2}, S Sarno^{1,2},
F Brustolon^{1,2}, LA Pinna^{1,2} and M Ruzzene^{*,1,2}

¹ Venetian Institute of Molecular Medicine (VIMM), University of Padova, Padova, Italy;

² Department of Biological Chemistry, University of Padova, Padova, Italy

* Corresponding author: M Ruzzene, Department of Biological Chemistry, University of Padova, V.le G. Colombo, 3 – 35121 Padova, Italy.

Tel: +39-049-8276112; Fax: +39-049-8073310;

E-mail: maria.ruzzene@unipd.it

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Abstract

Treatment of Jurkat cells with specific inhibitors of protein kinase CK2 induces apoptosis. Here we provide evidence that the antiapoptotic effect of CK2 can be at least partially mediated by upregulation of the Akt/PKB pathway. Such a conclusion is based on the following observations: (1) inhibition of CK2 by cell treatment with two structurally unrelated CK2 inhibitors induces downregulation of Akt/PKB, as judged from decreased phosphorylation of its physiological targets, and immunoprecipitate kinase assay; (2) similar results are observed upon reduction of CK2 catalytic subunit by the RNA-interference technique; (3) Akt/PKB Ser129 is phosphorylated by CK2 *in vitro* and *in vivo*; (4) such a phosphorylation of activated Akt/PKB correlates with a further increase in catalytic activity. These data disclose an unanticipated mechanism by which constitutive phosphorylation by CK2 may be required for maximal activation of Akt/PKB.

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Abbreviations: CK2, casein kinase 2; GSK3, glycogen synthase kinase 3; IQA, 5-oxo-5,6-dihydroindolo-(1,2-a)quinazolin-7-yl]acetic acid; MALDI, matrix-assisted laser desorption and ionization; MS/MS, tandem mass spectrometry; PDK1, 3-phosphoinositide-dependent protein kinase-1; PH, pleckstrin homology; PI3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B (Akt); PTEN, phosphatase and tensin homolog deleted on chromosome ten; RNAi, RNA interference; SGK, serum- and glucocorticoid-induced kinase; siRNA, short interfering RNA; TBB, 4,5,6,7-tetrabromobenzotriazole; WB, Western blot; w.t., wild type

Introduction

CK2, an acronym derived from the misnomer ‘casein kinase 2’, denotes a ubiquitous, essential and constitutively active

protein kinase affecting phosphoacceptor sites that are specified by numerous acidic residues (either Glu/Asp or phosphorylated aminoacids), among which the one at position n + 3 plays the most crucial role.¹

The high basal activity of the catalytic subunits (α and/or α') of CK2, either alone or in combination with a dimeric regulatory β -subunit to give an heterotetrameric holoenzyme, may reflect the striking pleiotropy of this kinase, whose present repertoire of substrates includes >300 proteins.¹ These are implicated in a wide variety of cellular functions, including global processes as well as signaling pathways, a circumstance that could account for the requirement of active CK2 both in quiescent and in stimulated cells.

CK2 is invariably elevated in tumors as compared to normal tissues (reviewed in Guerra and Issinger² and Tawfic *et al.*³) and unscheduled expression of its catalytic subunits is causative of transformation, especially under conditions that also alter the expression of oncogenes and tumor suppressor genes.^{4–6} Evidence has recently been accumulating that CK2 plays a prominent role in ensuring cell survival^{7,8} (and reviewed in Litchfield⁹) by counteracting apoptosis,^{10,11} a behavior which may account, at least in part, for its oncogenic potential. The mechanism(s), however, by which CK2 exerts its antiapoptotic role remain largely unexplored.

Evidence for the antiapoptotic role of CK2 has been obtained in LNCaP prostate cancer cells¹⁰ and in leukemia Jurkat T cells,¹¹ both lacking the phosphatase and tensin homolog deleted on chromosome ten (PTEN) protein^{12,13} which removes phosphate from the D3 position of inositol phospholipids, and which is also one of the numerous substrates of CK2.¹⁴ Lack of PTEN maintains the phosphatidylinositol 3-kinase (PI3-kinase) pathway in a constitutively active state. Therefore, in both LNCaP and Jurkat cell lines, as in many leukemias and lymphomas where PTEN is also absent, protein kinase Akt (also known as PKB), whose activation is promoted by the 3'-phosphoinositide-dependent kinase-1 (PDK1), is constitutively upregulated (for reviews on Akt/PKB see Hanada *et al.*,¹⁵ Scheid and Woodgett,¹⁶ and Nicholson and Anderson¹⁷). Akt exerts important antiapoptotic features, a property which is supposed to contribute to the tumor phenotype of PTEN-null cell lines.¹⁸

Interestingly, apoptosis of Jurkat cells can be promoted by two structurally unrelated specific inhibitors of CK2, which are inactive on either Akt or PDK1.^{19,20} This means that either CK2 operates through a pathway which is independent of Akt, or it can induce a hyperactivation of Akt beyond the level of constitutive activity displayed by this kinase in Jurkat and other PTEN-null cells.

To discriminate between these possibilities and to gain information about the apoptotic signaling pathways that might be affected by CK2, we have investigated the consequences of attenuating CK2 activity on the activity of Akt.

Here we provide evidence that *in vitro* and *in vivo* Akt is phosphorylated by CK2 at Ser129, with a concomitant increase in catalytic activity, thus disclosing the possibility

that constitutive phosphorylation by CK2 is required for full activation of Akt.

Results

CK2 inhibitors induce downregulation of Akt

In order to gain information about the mechanism by which CK2 exerts its antiapoptotic potential, we checked whether treatment of Jurkat cells with two selective inhibitors of CK2, 4,5,6,7-tetrabromobenzotriazole (TBB) and 5-oxo-5,6-dihydroindolo-(1,2-a)quinazolin-7-yl]acetic acid (IQA), had any effect on endogenous Akt activity. For this purpose, the phosphorylation level of Akt-specific targets was determined using phospho-specific antibodies. As a positive control for Akt inhibition, we treated cells with LY294002, a well-known inhibitor of PI3-kinase. Under our conditions, while LY294002 was totally ineffective on CK2 endogenous activity, both TBB and IQA markedly inhibited CK2 (^{11,20} and Figure 1a), and, as expected,^{11,20} induced cell death by apoptosis, as monitored by MTT viability assay (not shown) and by the cleavage of the caspase substrate PARP (Figure 1b). As far as the Akt signaling pathway is concerned, treatment of Jurkat cells with both CK2 inhibitors resulted in a marked drop in the phosphorylation level of GSK3 β Ser9, FKHR Ser256, and AFX Ser193 (Figure 1c). Note that phosphorylation of PDK1, the upstream kinase responsible for canonical Akt regulation, is not altered by either TBB or IQA. Consequently, also phosphorylation of Akt Thr308, which is specifically targeted by PDK1, is only marginally reduced. A weak inhibition of Ser473 phosphorylation is also observed; this is negligible as compared to that promoted by the PI3-kinase inhibitor LY294002, and therefore not sufficient to justify the reduction of Akt activity monitored by the drop in substrate phosphorylation. Figure 1c also shows that the total amount of Akt and glycogen synthase kinase 3 (GSK3) proteins was not affected by the treatments.

We also measured directly the activity of Akt towards a synthetic peptide in an immunoprecipitate kinase assay, after cell incubation with the CK2 inhibitors. As shown in Figure 2, the catalytic activity of Akt was significantly reduced by treatments with both inhibitors, IQA and TBB, under conditions where comparable amounts of enzyme were present in the immunoprecipitates.

Decreased levels of CK2 catalytic subunit correlate with inactivation of Akt

In order to make sure that the detrimental effect of CK2 inhibitors on the activity of Akt was really mediated by blockage of CK2, rather than of other kinases, advantage has been taken of the RNA interference (RNAi) technique to achieve a nonpharmacological and strictly selective decrease in endogenous CK2 catalytic activity. LNCaP cells were used for these experiments (given the availability of simpler protocols to trigger RNA interference in adherent than in suspension cells). Similar to Jurkat, LNCaP cells are PTEN-null and therefore Akt is constitutively activated. As shown in Figure 3a, the approach proved successful for diminishing the level of expression of CK2 α catalytic subunit to about 39%.

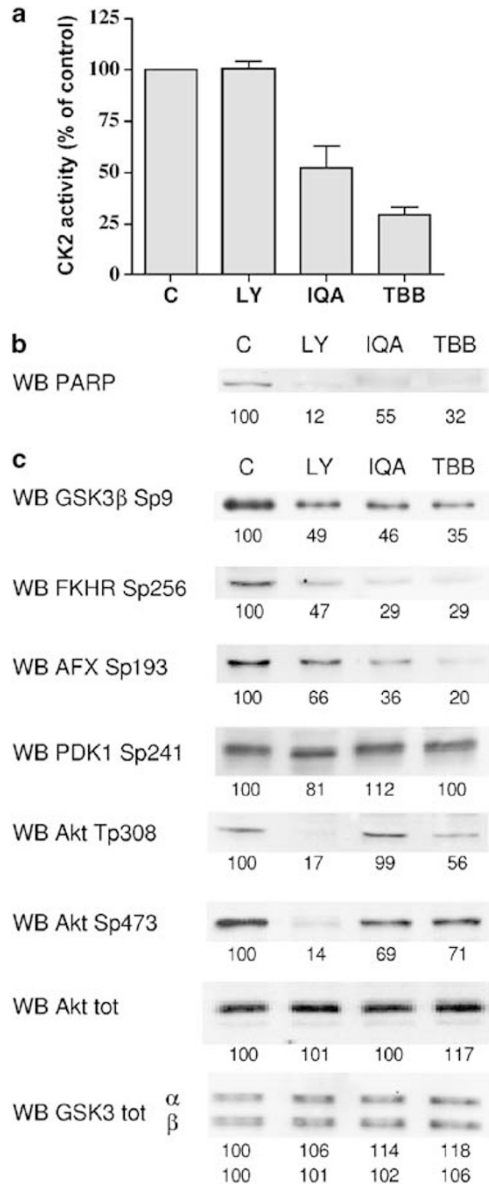


Figure 1 Downregulation of Akt pathway by CK2 inhibitors in Jurkat cells. Jurkat cells were treated for 4 h with CK2 inhibitors, as indicated. IQA was 40 μ M, TBB was 25 μ M. Where shown (LY), the PI3-kinase inhibitor LY294002 (25 μ M) was used as a control for Akt downregulation. (a) Activity of CK2 in cell lysates was measured towards the CK2-specific peptide substrate (bar graphs, mean values (\pm S.D.) of three determinations). In all, 100% CK2 activity was assigned to lysate from control cells (C, 0.15 pmol/min/ μ g protein). (c and b) 5 μ g (in the case of anti-Akt Sp473) or 20 μ g (for all the other antibodies) of total lysate proteins were analyzed by Western blot (WB) with the indicated antibodies. Band quantitation was obtained on a Kodak Image Station 440CF by analysis with the Kodak 1D Image software, and the values are reported below each band, assigning the value of 100 to the control cells (C)

This reduction results, as expected, in a substantial drop in CK2 catalytic activity (of about 36%). As shown in Figure 3b, the knock down of the CK2 catalytic subunit is accompanied by a substantial reduction of Akt activity, as judged by immunoprecipitate kinase assay, or by the dephosphorylation degree of the endogenous targets GSK3 β Ser9 and FKHR Ser256. Under these conditions, the GSK3 and Akt protein amounts were not affected, nor the Akt phosphorylation state

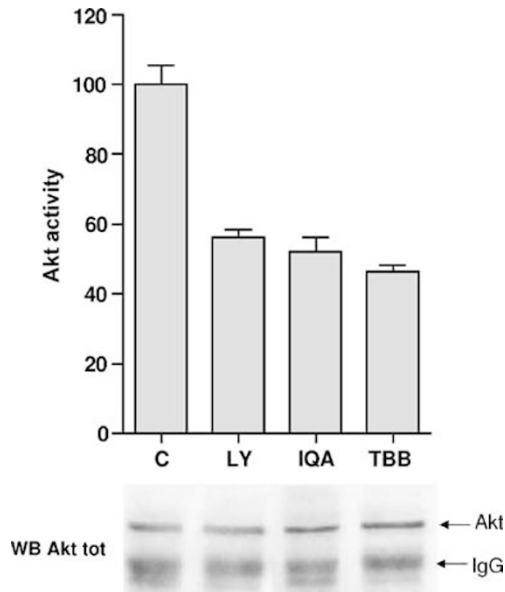


Figure 2 Akt immunoprecipitate kinase assay from Jurkat cells treated with CK2 inhibitors. Jurkat cells were treated for 4 h with CK2 inhibitors, as indicated. IQA was 40 μ M, TBB was 25 μ M. Where shown (LY), the PI3-kinase inhibitor LY294002 (25 μ M) was used as a control for Akt downregulation. Akt was immunoprecipitated from the cell lysate, and its activity was measured towards a peptide substrate, by incubation for 30 min in the presence of a radioactive phosphorylation mixture, as described in Materials and Methods; the radioactivity incorporated is reported as arbitrary units, assigning an activity of 100 to Akt immunoprecipitated from control cells (C). Mean values (\pm S.D.) from two separate experiments are shown. The amount of total Akt in each immunoprecipitate was monitored by Western blot, as shown in the bottom part of the figure

was significantly altered (not shown). This indicates that Akt activity is effectively downregulated in cells treated with short interfering RNA (siRNA) against CK2 catalytic subunit.

CK2 phosphorylates and activates Akt *in vitro*

The residues whose phosphorylation is known to be most responsible for Akt activation are Thr308 (in the activation loop) and Ser473 (in the hydrophobic domain outside the catalytic domain). The former is phosphorylated by PDK1,²¹ while Ser473 phosphorylation is still a matter of debate, one possibility being that this may occur autocatalytically. In any event, neither Ser473 nor Thr308 display the consensus required for CK2 phosphorylation, not to say that in PTEN-null cells both these residues are expected to be constitutively phosphorylated, irrespective of CK2. Akt includes, on the other hand, a few residues that display the consensus sequence for CK2-mediated phosphorylation, and we wanted to see if the *in vivo* activation of Akt by CK2 activity might be due, at least in part, to direct phosphorylation of Akt by CK2. To check this possibility, a number of *in vitro* experiments were performed where variably phosphorylated forms of Akt were subjected to phosphorylation by CK2. The results are shown in Figure 4. It can be seen that both an active form of Akt, previously phosphorylated at both Ser473 and Thr308, and an inactive unphosphorylated form are readily phosphorylated by the CK2 holoenzyme, and, even more, by CK2 α (compare lanes 2 and 5 with lanes 3 and 6, respectively).

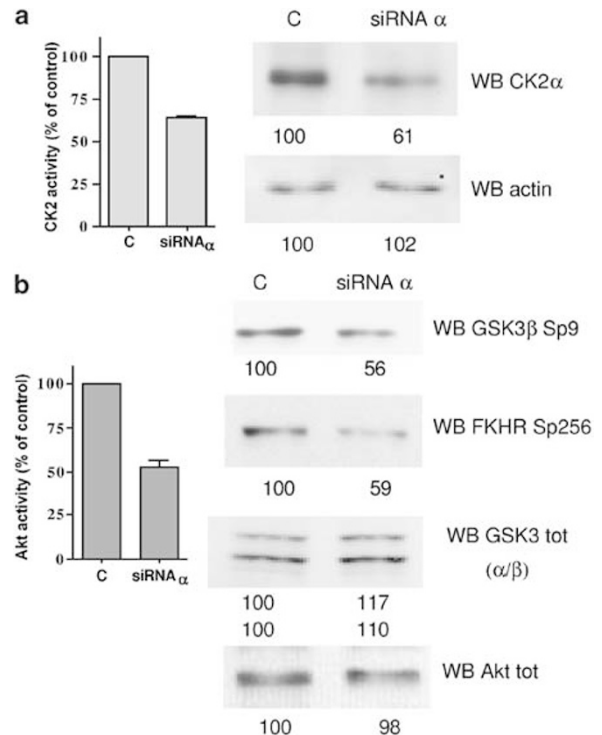


Figure 3 Knock down of CK2 by the RNA interference technique and effect on Akt signaling. LNCaP cells were treated for 72 h with siRNA sequences targeting CK2 α . When Western blot experiments are shown, band quantitation is reported below each band, assigning the value of 100 to control cells (C), treated with non silencing siRNA. (a) The bar graph shows the activity of CK2 in cell lysates, measured towards the CK2-specific peptide substrate (mean values (\pm S.D.) of two determinations). 100% CK2 activity was assigned to lysate from control cells. The right part of the figure shows the Western blot (10 μ g of total proteins) with anti-CK2 α specific antibodies or antiactin, for normalization. (b) The bar graph shows the activity of Akt, measured towards a peptide substrate, after immunoprecipitation from cell lysates (mean values (\pm S.D.) of two determinations). In all, 100% Akt activity was assigned to the Akt immunoprecipitated from control cells. The right part of the figure shows the Western blot (10 μ g of total proteins) with the indicated antibodies

Interestingly, three related kinases with remarkable similarity to Akt, but lacking consensus sequences for CK2, serum- and glucocorticoid-induced kinase (SGK)-1, 2 and 3, are not phosphorylated by CK2 to any appreciable extent (not shown).

We wondered whether phosphorylation by CK2 might have any effect on Akt activity. We therefore performed experiments in which the activity of Akt, incubated or not with CK2 and Mg/ATP, was determined using either a peptide (Figure 5a) or a protein (Figure 5b) substrate, containing the consensus for Akt (R/K-x-R/K-x-x-S/T) but not for CK2. The results demonstrate that CK2 promotes a ~2-fold hyperactivation of active Akt and that this effect is mediated by phosphorylation, as it is suppressed by the CK2-specific inhibitor TBB.

In the absence of previous phosphorylation at Thr308 and Ser473, Akt activity was undetectable with the peptide/protein substrates used, either before or after phosphorylation by CK2 (not shown); however, a weak signal was detected using the anti-phospho-Ser473 antibodies, and this was significantly

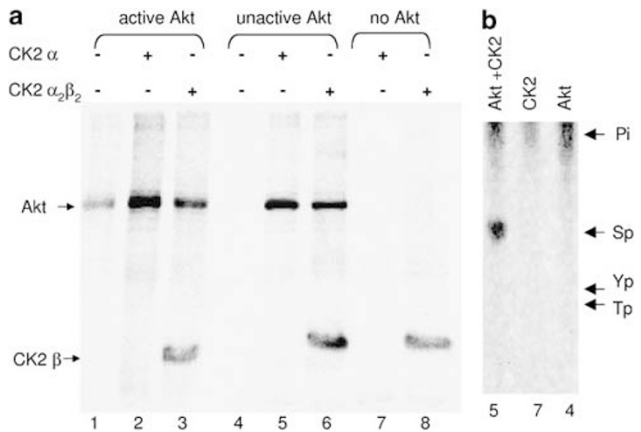


Figure 4 *In vitro* phosphorylation of Akt by CK2. Incubations were performed for 20 min in the presence of radioactive phosphorylation mixture (see Materials and Methods), with the indicated protein kinases. In all, 0.2 μ g of active or inactive Akt1 was used. Under the conditions used, the CK2 α (0.1 μ g) or CK2 $\alpha_2\beta_2$ (0.02 μ g) amounts were equally active towards casein substrate (not shown). (a) An autoradiography is shown of phospho-proteins resolved on SDS/PAGE. Autophosphorylation of CK2 β subunit is also visible. Stoichiometry of Akt1 phosphorylation was about 0.2 mol phosphate/mol protein. (b) Autoradiography of the high-voltage paper electrophoresis for the identification of the phospho-amino acid. Samples from lanes 4, 5, and 7 of panel a were hydrolyzed in 6 N HCl, and subjected to high-voltage paper electrophoresis, as described in Material and Methods, for the identification of the phospho-amino acid. An autoradiography of the paper is shown. The position of orthophosphate (Pi) and phospho-amino acids (Sp, Yp, and Tp), determined by cold standard migration, is indicated by the arrows

increased by CK2 (Figure 5c). Since the Ser473 site is not affected by CK2 (see below), this finding must reflect a CK2-induced stimulation of Akt autophosphorylation activity.

Ser129 is the major target for CK2 in Akt

Akt1 contains six residues that fulfill the consensus sequence for CK2 (S/T-x-x-E/D), namely Thr92, Ser129, Thr146, Thr165, Ser259, and Ser431. All of these, with the exception of Ser129, are 'poor' phosphoacceptor sites, for having no additional acidic residues in the proximity of the target amino acids, a part from the one at position $n+3$.¹ Conversely, Ser129 can be classified as a good CK2 target for having acidic residues at positions $n+3$, $+4$, $+6$, and -2 . Moreover, once phosphorylated it could prime the phosphorylation of Ser126.

Phosphorylation of threonyl residues was ruled out by phosphoaminoacid analysis showing that only phosphoserine could be isolated from Akt1 phosphoradiolabeled by CK2 (Figure 4b).

In order to identify the seryl residue(s) phosphorylated by CK2, recombinant Akt1 previously phosphorylated *in vitro* by CK2 was subjected to SDS/PAGE and its band was digested with trypsin in gel and analyzed by matrix-assisted laser desorption and ionization (MALDI) mass spectrometry. As shown in Figure 6a, a peptide with a molecular weight compatible with the tryptic fragment [122–142] (2119.83 Da) was detected together with its possible monophosphorylated form (2199.83 Da). To prove that the peptide with the higher molecular mass was actually due to a phosphorylation of the

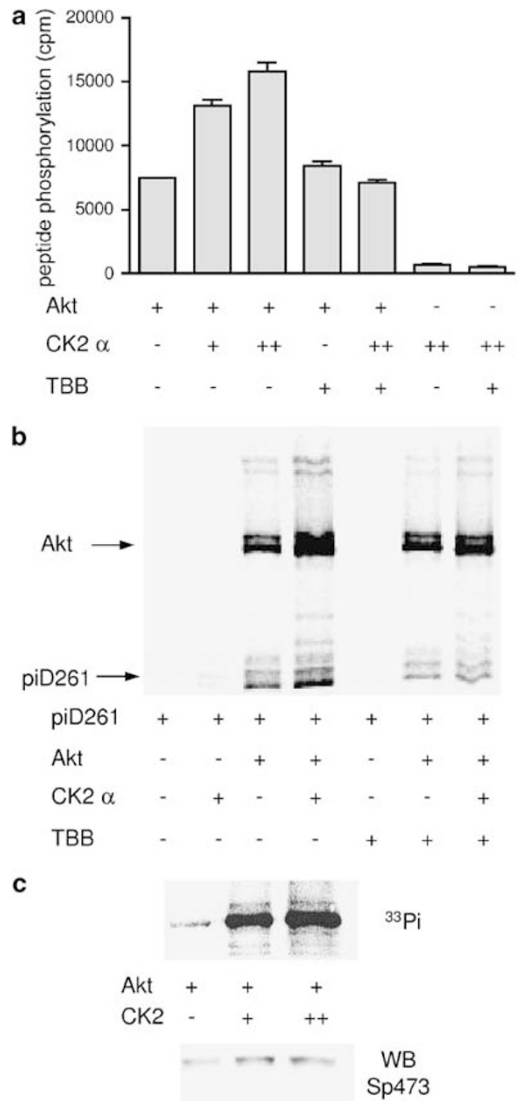


Figure 5 Effect of CK2 on the activity of Akt *in vitro*. (a) Active Akt (0.2 μ g) was incubated for 20 min with radioactive phosphorylation mixture in the absence (–) or in the presence of 0.05 μ g (+) or 0.1 μ g (++) CK2 α . Where indicated, TBB was present at 1 μ M concentration. Akt activity measured towards a peptide substrate is shown as cpm incorporated (mean values \pm S.D.) from three separate experiments (b) Active Akt (0.2 μ g) was incubated as described for panel a, but its activity was evaluated towards the protein substrate piD261 (0.1 μ g); an autoradiography is shown of phospho-proteins resolved on SDS/PAGE. (c) Unactive Akt (0.2 μ g) was incubated for 20 min with radioactive phosphorylation mixture in the absence (–) or in the presence of 0.05 μ g (+) or 0.1 μ g (++) CK2 α ; the incorporated radioactivity is shown in the upper part, while the degree of Ser473 phosphorylation detected by Western blot with the phospho-specific antibody is shown in the bottom part

peptide [122–142], the sample was chemically dephosphorylated and analyzed again by MALDI mass spectrometry. As shown in Figure 6b, the peak underwent the 98 Da shift expected for β -elimination of phosphate from seryl/threonyl residue. The [122–142] sequence includes the best potential CK2 site (Ser129) together with other four seryl residues. In order to determine the exact phosphorylation site, a tandem mass spectrometry (MS/MS) spectrum of the phosphopeptide

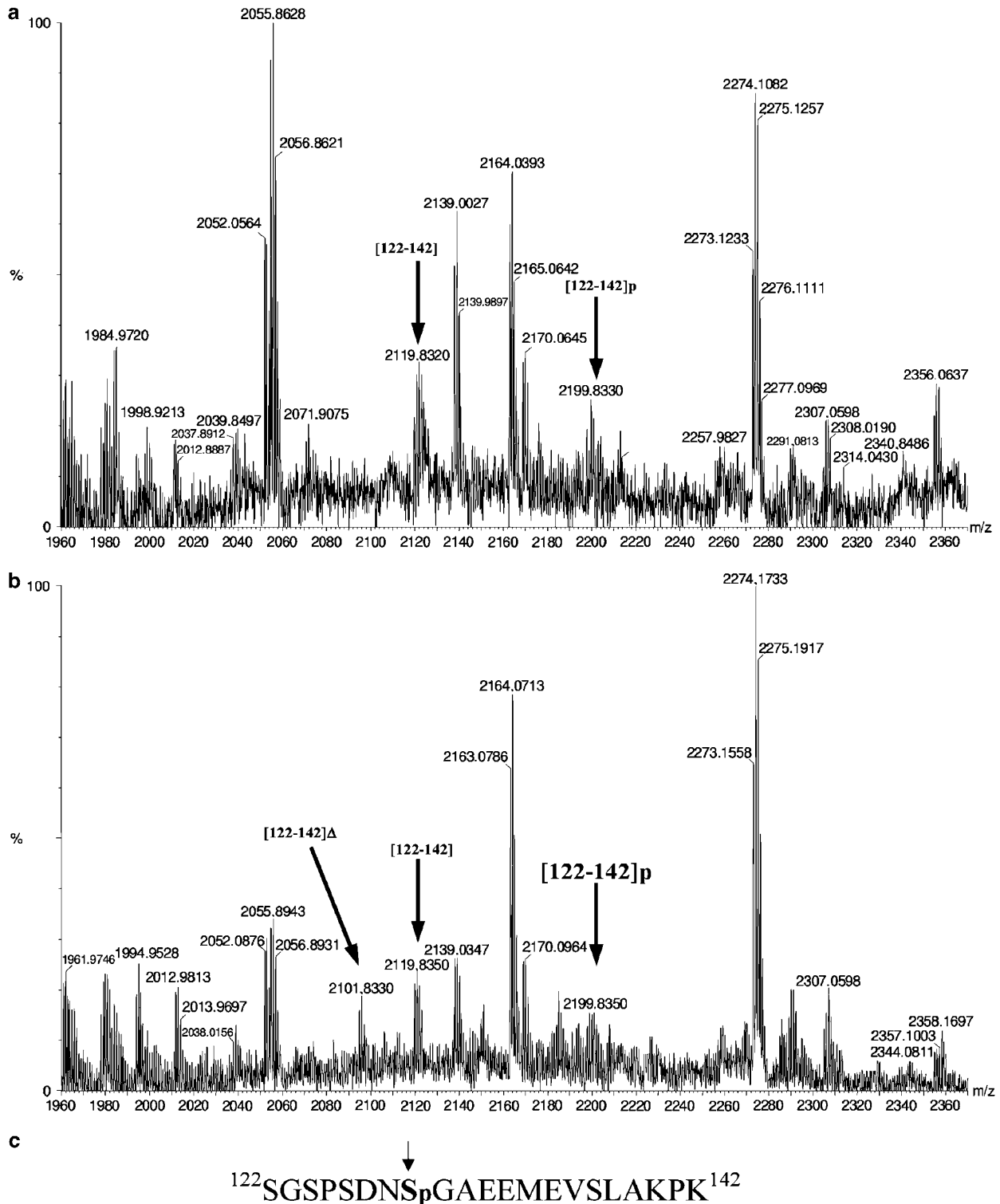


Figure 6 Identification of the CK2 phosphorylation site on Akt. **(a)** Portion of the MALDI spectrum of Akt1 phosphorylated by CK2 and digested with trypsin as described in Materials and Methods. The peaks corresponding to the tryptic peptide [122–142] (2119.83 Da) and its phosphorylated form [122–142]p (2199.8 Da) are indicated. **(b)** The same portion of the spectrum of panel (a), after treating the sample with Ba(OH)₂. A new peak, indicated as [122–142]Δ (2101.83 Da), is now evident: it corresponds to the chemically dephosphorylated [122–142] peptide, having a mass shift of 98 Da with respect to the phosphorylated product, due to the β-elimination of the phosphate group. **(c)** Sequence of human Akt1 [122–142]. Ser129, target of CK2, is indicated

[122–142] was acquired (not shown). The manual interpretation of the spectrum allowed us to identify Ser129 as the phosphorylated residue.

To confirm the identification of Ser129 as the major target for CK2 in Akt1, we mutated this serine to alanine, and overexpressed both the mutant S129A Akt and the wild-type

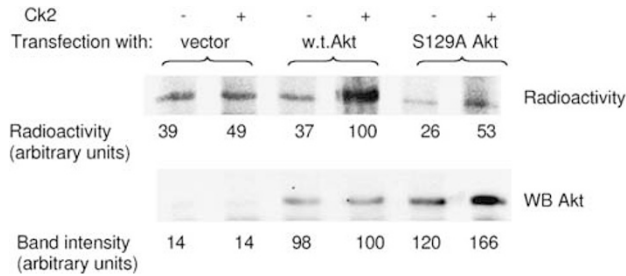


Figure 7 Impaired phosphorylation of the Ser129 Akt mutant by CK2. HEK 293T cells were transfected with vector alone, w.t. Akt, or S129A Akt mutant, as indicated. Akt was immunoprecipitated from cell lysates, then incubated in the presence of radioactive phosphorylation mixture, with the addition of 0.1 μ g of CK2 α , where indicated (+). Samples were then loaded on SDS/PAGE followed by blotting, and analyzed by autoradiography (a) or Western blot with anti-Akt antibodies (b). The values reported below each band represent the radioactivity (a), or the intensity of the ECL-developed band (b), and are expressed in arbitrary units

(w.t.) Akt in the highly transfectable HEK 293T cell line, reaching a very similar overexpression level in both cases (not shown). We performed experiments where equal amounts of w.t. Akt and S129A Akt were immunoprecipitated and used as substrates for recombinant CK2 in an *in vitro* phosphorylation assay. As shown in Figure 7, while w.t. Akt, as expected, was readily phosphorylated by CK2, the phosphorylation of the S129A mutant was severely impaired.

Ser129 is phosphorylated *in vivo*

To assess if Ser129 of Akt is phosphorylated in cells, we developed phospho-specific antibodies towards a synthetic phospho-peptide corresponding to amino-acid residues [121–140] of human Akt1 sequence, and containing phospho-Ser129. The antibodies were first checked in Western blot experiments with the recombinant Akt, phosphorylated or not by CK2 *in vitro*. The results, shown in Figure 8a, highlight the specificity of the antibodies towards the phosphorylated form, thus confirming the identification of Ser129 as the target site for CK2 *in vitro*. When Jurkat cell lysates were analyzed with these antibodies, a weak signal was detected, which became lower in cells treated with TBB (Figure 8b). The response evoked in Western blot by the phospho-Ser129 antibodies was obviously stronger in HEK 293 T cells transfected with w.t. Akt (Figure 8b); also in this case the signal was significantly decreased by TBB treatment of the cells, while it was absent in cells where the S129A Akt mutant was transfected. In contrast, the signal evoked by an antibody raised against the same region but with nonphosphorylated Ser129 was unchanged.

Parallel experiments were performed by mass spectrometry analysis of the Akt band immunoprecipitated from cells transfected with w.t. Akt: the gel band was treated as described in the Material and Methods section for the in-gel tryptic digestion and analyzed by mass spectrometry. The presence of a phosphorylated form of the peptide [122–142] was confirmed acquiring MALDI MS spectra of the tryptic digest. Again, the acquisition of MS/MS spectra allowed us to confirm the presence of Ser129 as a phosphorylated residue (data not shown).

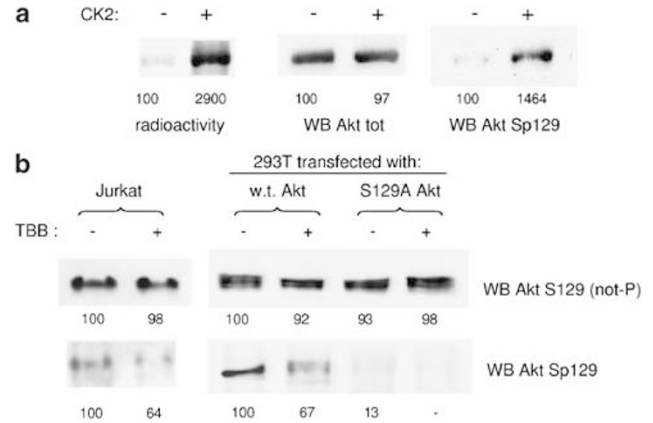


Figure 8 Evidence for Akt Ser129 phosphorylation *in vivo*. (a) Active Akt (0.05 μ g) was incubated for 20 min with radioactive phosphorylation mixture in the absence (–) or in the presence (+) of 0.02 μ g CK2 α . The radioactivity detected by autoradiography, or the signal obtained by Western blot with specific antibodies, are shown, as indicated. (b) Lysates from Jurkat cells or from HEK 293T cells (transfected with w.t. Akt, or S129A Akt mutant) were analyzed by Western blot with the indicated antibodies. In all, 40 or 10 μ g of total proteins was loaded, in the case of Jurkat or HEK 293T cells, respectively. Where indicated, TBB (50 μ M) cell treatment was performed during the last 3 h before cell lysis. Quantitation of the bands is reported below each band, expressed in arbitrary units, assigning 100 to not treated Jurkat cells, and to not treated 293T cells transfected with w.t. Akt

Since HEK 293T cells do express significant amounts of CK2 (not shown), and considering the decrease of Ser129 phosphorylation observed upon inhibition of CK2 (Figure 8b), we conclude that the w.t. Akt overexpressed from 293 T-cell lysate is phosphorylated at Ser129 *in vivo* by CK2, although, probably, not stoichiometrically, since it can be further phosphorylated by CK2 *in vitro* at the same site (see above, Figure 7).

The *in vivo* activity of the S129A Akt mutant is impaired

Next we wanted to test if the activity of Akt overexpressed in HEK 293 T cells was impaired by the mutation of Ser129 to Ala. To this purpose, we immunoprecipitated w.t. and S129A Akt and tested their activity towards the Akt peptide substrate. We found that the activity of S129A Akt mutant is markedly reduced, compared to the w.t. enzyme (Figure 9). We also analyzed by Western blot the *in vivo* phosphorylation state of the Akt substrate GSK3 β , and of Thr308 and Ser473 of Akt itself, in cells transfected with either w.t. or S129A Akt. We observed a significant reduction of GSK3 phosphorylation in cells transfected with the mutant (Figure 9b). The mutation of Ser129 to Ala also promoted a slight decrease of Ser473 and Thr308 phosphorylation, not sufficient *per se* to justify the drop in Akt activity.

Discussion

The data presented disclose a novel functional connection between CK2 and Akt, by showing that, in living cells, downregulation of CK2 catalytic activity or mutation of the

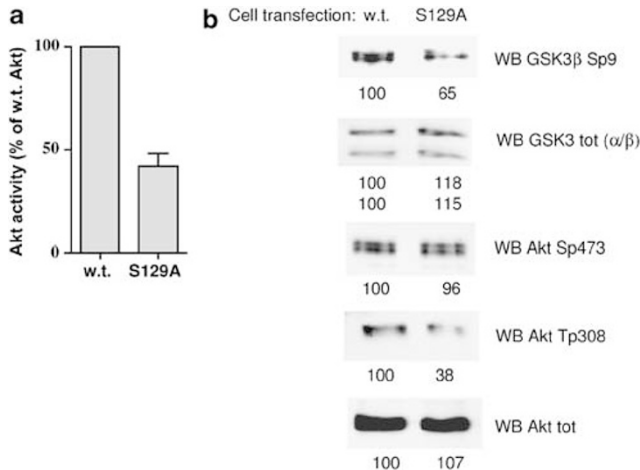


Figure 9 Akt activity in cells transfected with w.t. Akt or Ser129 Akt mutant. HEK 293T cells were transfected with w.t. Akt or S129A Akt mutant, as indicated. (a) Akt was immunoprecipitated from cell lysates, and its activity was measured towards a peptide substrate, by incubation for 30 min in the presence of a radioactive phosphorylation mixture. The peptide phosphorylation is reported as arbitrary units, assigning an activity of 100 to Akt immunoprecipitated from cells transfected with w.t. Akt. Mean values (\pm S.D.) from two separate experiments are shown. (b) In all, 5 μ g (in the case of anti-Akt Sp473) or 20 μ g (for all the other antibodies) of total lysate proteins was analysed by Western blot (WB) with the indicated antibodies. Band quantitation (arbitrary units, assigning 100 to the signal from cells transfected with w.t. Akt) is reported below each band

CK2-dependent site in Akt correlates with decreased Akt activity, as judged from reduced *in vivo* phosphorylation of typical Akt targets, and from immunoprecipitate kinase assay.

These data are remarkable inasmuch as they were obtained with PTEN-null cells (either Jurkat or LNCaP) where Akt is already constitutively active and where the possibility is ruled out that CK2 might operate through PTEN, whose *in vitro* and *in vivo* phosphorylation by CK2 has been reported previously.¹⁴ Also note, in this respect, that the CK2 inhibitors used in this study are ineffective on all isoforms of PI3-kinase²⁰ (and unpublished), and they do not affect the activation state of PDK1, supporting the view that CK2 does not impinge on upstream events leading to Akt activation. A direct effect of CK2 on Akt is actually supported by *in vitro* experiments showing that Akt can be phosphorylated by CK2 with an increase in catalytic activity. Such a stimulation of activity by CK2 could be even more pronounced than it appears from our experiments (Figure 5), considering that *in vitro* phosphorylation is understoichiometric (see legend of Figure 4).

As expected, CK2 does not phosphorylate Akt at the residues previously reported to trigger Akt activation, notably Thr308 and Ser473, neither of which displays the consensus for CK2. In contrast, CK2 phosphorylates Ser129, a residue located in the linker region between the pleckstrin homology (PH) and the catalytic domains. Ser129 displays the features of a primary target for CK2 (for having an acidic residue at position $n+3$), and its actual phosphorylation has been documented by MS/MS analysis. Ser129 has also been confirmed as the major CK2 target in Akt by its mutation to alanine, which generates a form almost completely unaffected by CK2. Once phosphorylated, Ser129 may trigger the subsequent phosphorylation of Ser126, with a hierarchical mechanism found in several CK2 sites.¹ Consequently,

mutation of Ser129 would also abrogate Ser126 phosphorylation. It can be concluded, therefore, that phosphorylation of Akt at Ser129 (and possibly Ser126) is responsible for hyperactivation of Akt by CK2. Note that Ser129 is conserved in both Akt1 and Akt2. It is not conserved, however, in the related protein kinases SGK, which are not phosphorylated *in vitro* by CK2.

An *in vivo* role of CK2-mediated phosphorylation of Akt is strongly supported by data obtained with cells transfected with w.t. or S129A mutant Akt, where the Ser129 phosphorylation is shown to occur in living cells and to correlate with increased activity of Akt.

Pertinent to this may be also the finding that Akt overexpressed in 293 cells contained phosphate constitutively present in the peptide [122–140];²² interestingly, this peptide eluted as a doublet from C18 chromatography, suggesting the presence of multiple phosphorylated forms; indeed, besides the major phospho-aminoacid, identified as Ser124, a weak signal at Ser129 was also reported. While CK2 phosphorylates equally well *in vitro* either inactive or activated Akt1, its favorable effect on activity is evident only with active Akt1, whereas the activity of inactive Akt remains negligible also after phosphorylation by CK2. It may be worth to note, however, that the weak autophosphorylation of inactive Akt1 at Ser473 is significantly enhanced by previous phosphorylation with CK2, supporting the view that this latter event induces anyway conformational changes rendering Akt more prone to activation. Collectively taken, our data suggest that constitutive phosphorylation of Akt by CK2 gives rise to a pool of kinase, which is susceptible to become hyperactive upon phosphorylation of Thr308 by PDK1. Our *in vivo* experimentation with S129A Akt mutant was performed in a system where Akt is expressed as constitutively active; we therefore cannot discriminate at which level CK2 exerts its role, either before or after canonical activation of Akt triggered by Thr308/Ser473 phosphorylation.

Another point which will deserve additional attention is the weak dephosphorylation of Akt Ser473 and Thr308 observed in cells where CK2 is downregulated. At present, our data do not provide an explanation for this observation; however, it is worth to consider the connection to the Hsp90/cdc37 chaperone system: this complex, known to protect Akt from dephosphorylation,²³ has been recently demonstrated to be fully competent for its action only if phosphorylated by CK2.²⁴ Therefore, this could be a different level of crosstalk between CK2 and Akt. Further studies will be necessary to clarify this issue.

Does stimulation of Akt activity account for the antiapoptotic role of CK2? A definite answer to this question is still premature. On the one hand, there is no doubt that an increment of Akt activity would represent a means to counteract apoptosis, given the central role of this kinase to ensure cell survival. Consistent with this, a Jurkat cell line in which Akt is overexpressed is significantly more resistant to apoptosis induced by CK2 inhibitors than are control Jurkat cells (unpublished), supporting the view that the antiapoptotic effect of CK2 is mediated, at least in part, by Akt.

On the other hand, there is no evidence that stimulation of Akt activity represents the only mechanism by which CK2 promotes cell survival. It should be emphasized that, given its

extreme pleiotropy, CK2 can impinge on numerous pathways, eventually cooperating with Akt signaling to counteract apoptosis. For example, phosphorylation by CK2 of I κ B in its PEST region accelerates I κ B degradation by calpain,^{25–27} thus promoting the nuclear translocation of NF- κ B and increased expression of antiapoptotic genes. An even more general mechanism by which CK2 can display its antiapoptotic potential would be to render caspase substrates refractory to cleavage: numerous examples are already known of proteins (e.g. Max, Bid, HS1, connexin 45.6, and PTEN) whose phosphorylation by CK2 hinders their cleavage by caspases²⁸ (and reviewed in Litchfield⁹ and Pinna²⁹). But the connection between CK2 and caspases could be even tighter. Besides counteracting the effects of caspases downstream, at substrate level, an upstream device by which CK2 turns off caspase activity has recently been reported,³⁰ consisting of the phosphorylation of the caspase-inhibiting protein ARC. Such a phosphorylation is required to target ARC to mitochondria, where it can bind (and inhibit) caspase 8. Another mechanism by which CK2 may prevent cell death has recently been reported;³¹ the authors show that the proapoptotic function of CHOP transcription factor is inhibited by CK2-mediated phosphorylation.

In the emerging scenario, the newly detected connection with Akt would be a part of a multifaceted strategy by which CK2 coordinates a network of signaling pathways leading to cell survival. Note that some of these, notably caspase inhibition and I κ B degradation, could be promoted independently by both CK2 and Akt.

Materials and Methods

Materials

Antibodies

Phospho-GSK3 β (S9), phospho-FKHR (S256), which also recognized phospho-AFX (S193), phospho-Akt (T308), and phospho-PDK1 (S241) antibodies were from Cell Signaling Technology, phospho-Akt (S473), total Akt1/2/3, total GSK3 β and actin antibodies were from Santa Cruz Biotechnology, CK2 α -subunit rabbit antiserum was raised against [376–391] region of human protein, corresponding to the specific C-terminal sequence. Phospho-Akt (S129) and its not-phosphorylated counterpart (Akt (S129)) were raised in New Zealand rabbits against two synthetic peptides corresponding to amino acids [121–140] of the Akt protein, phosphorylated in Ser129 or not, respectively; the antisera were purified using an immobilized peptide affinity resin (Sulfo Link Coupling Gel, Pierce, UK) according to the manufacturer's instructions

Kinase substrates

CK2-specific peptide substrate was synthesized as described elsewhere.³² Akt-specific peptide substrate was from Calbiochem, Akt protein substrate piD261 was expressed, purified³³ and kindly provided by Dr. S Facchin (Padova).

Inhibitors

TBB was synthesized as in Szyska *et al.*,³⁴ IQA, formerly termed CGP029482,²⁰ was provided by Novartis; LY294002 was from Cell Signaling Technology, PKA inhibitor peptide was from Sigma.

cDNA constructs

The expression vector for the murine HA-tagged myrAkt1 (pCMV6-HA-myrAkt1), a constitutively active Akt containing a consensus myristoylation site, was kindly supplied by Dr. Sandra Marmioli (Modena). S129A Akt mutant was obtained with the 'QuickChange-Site Directed Mutagenesis' Kit (Stratagene) using pCMV6-HA-myrAkt1 as template and two synthetic oligonucleotides primers, 5'-CCCAGTGACAACGCAGGGGCTGAAGAGATGG-3' and 5'-CCATCTCTTCATCCCCTGCGTTGTCTACTGGG-3' each complementary to opposite strands of template. The mutation was confirmed by sequencing analysis.

Enzymes

Active full-length Akt1 (phosphorylated at Thr308 and Ser473) and inactive full-length Akt1 (dephosphorylated) were provided by Dr. H McLauchlan and Dr. J Hastie (Dundee), recombinant CK2 α and $\alpha_2\beta_2$ were expressed and purified as in Sarno *et al.*¹⁹

Cell culturing and treatment

The human leukemia Jurkat T-cell line, the human prostate cancer LNCaP cell line, and the human embryonic kidney epithelial HEK 293T cells were maintained in the appropriate medium (RPMI 1640 (Dutch modification, Sigma) for Jurkat and LNCaP, Dulbecco's modified Eagle's medium (Sigma) for HEK 293T, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, in an atmosphere containing 5% CO₂.

Cell treatments were performed as elsewhere described.¹¹

Cell lysis and Western blot analysis

After treatment, cells were centrifuged, washed, and lysed by the addition of ice-cold buffer consisting of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% (v/v) Triton X-100, 2 mM dithiothreitol, protease inhibitor cocktail Complete (Roche), 10 mM NaF, 1 μ M okadaic acid, 1 mM Na vanadate. After 20 min incubation on ice, the lysates were centrifuged at 10 000 \times g for 10 min, at 4°C. The supernatants contained the cell-soluble fraction, whose protein concentration was determined by the Bradford method. Equal amounts of protein were loaded on 11% SDS/PAGE, blotted on Immobilon-P membranes (Millipore), and processed in Western blot with the indicated antibody, detected by ECL (enhanced chemiluminescence, Amersham Biosciences). Quantitation of the signal was obtained by chemiluminescence detection on a Kodak Image Station 440CF and analysis with the Kodak 1D Image software.

siRNA method

The siRNA sequence targeting specifically human CK2 α containing 21-nt with (2'-deoxy)-thymidine 3' overhangs and corresponding to nucleotides 803–823 (Accession Number AY112721) was synthesized and annealed by Qiagen-Xeragon Inc. The day before the transfection, cells were diluted with fresh medium without antibiotics and transferred to six-well plates. LNCaP cell transient transfection of siRNA was carried out according to Elbashir *et al.*,³⁵ using Oligofectamine (Invitrogen). In all, 12 μ l culture medium and 3 μ l Oligofectamine per well were preincubated 5 min at room temperature. During the time of this incubation, 175 μ l medium were mixed with 10 μ l siRNA. The two mixtures were combined and incubated for 20 min at room temperature for complex formation. The entire mixture (200 μ l) was added to the cells in one well resulting in a final concentration

of 100 nM for the siRNA. Control cells were treated with 100 nM control nonsilencing siRNA (Qiagen).

Cell transfection

HEK-293T cells, plated onto 60-mm-diameter dishes at about 80% confluency, were transiently transfected with 4 μ g of cDNA by standard calcium-phosphate procedure. The transfection mixture was removed after 16 h, and cells were lysed after 48 h after transfection.

CK2 activity assay in cell lysates

In all, 1–2 μ g of lysate proteins were incubated for 10 min at 30°C with 1 mM CK2-specific peptide RRRADSDDDDD, in the presence of phosphorylation reaction mixture.¹¹

In vitro phosphorylation of Akt by CK2

Purified recombinant Akt (0.2–0.5 μ g) was incubated with recombinant CK2 (α or $\alpha_2\beta_2$, 10–20 units, where 1 unit is the amount of enzyme transferring 1 pmol of Pi to the peptide substrate per min) in the presence of 50 mM Tris-HCl, pH 7.5, 12 mM MgCl₂, 10 μ M [γ -³²P]ATP (~1500 cpm/pmol), 0.1 M NaCl, in a total volume of 20 μ l, for 10 min at 30°C.

In the case of overexpressed Akt, Akt was immunoprecipitated from 40 μ g of lysate proteins with total Akt antibody, following the protocol described elsewhere;¹⁹ the immunoprecipitates were then incubated with CK2, as described for purified Akt.

Samples were run on 11% SDS/PAGE, and radioactive bands were detected and quantified by autoradiography (Cyclone storage phosphor system, Packard).

For the identification of the amino acids phosphorylated by CK2 in Akt, after phosphorylation samples were hydrolyzed by incubation for 4 h at 110°C in 6 N HCl, then subjected to high-voltage paper electrophoresis at pH 1.9 for 2.5 h, in the presence of nonradioactive phospho-amino acids, as migration reference.³⁶

Akt activity assay

For Akt immunoprecipitation, 250 or 40 μ g of total lysate proteins, in the case of Jurkat/LNCaP or HEK 293T cells, respectively, were incubated with total Akt antibody, following the protocol described elsewhere.¹⁹ Akt immunoprecipitate kinase assay was performed as described in Cross *et al.*,³⁷ towards 0.1 mM substrate peptide (RPRAATF), in the presence of 1 μ M PKA inhibitor peptide. To verify the specificity of the kinase reaction, the activity was also measured in the immunoprecipitates with equal amount of an aspecific antibody, where no Akt was revealed by Western blot analysis.

The activity of purified Akt (specific activity of 1.2 pmol Pi/min/ μ g, under basal conditions) was measured towards either the substrate peptide, or the recombinant yeast protein kinase piD261/Bud32,³³ which contains in its C-terminal tail a site conforming to the consensus sequence of Akt and which is not affected by CK2. Neither the peptide nor piD261 are phosphorylated by CK2 to any appreciable extent. Akt was incubated for 20 min at 30°C, with or without CK2, under the conditions described above, for Akt phosphorylation, with the addition of the substrate (0.1 mM peptide, or 0.1 μ g piD261 protein). The substrate phosphorylation degree was detected by means of SDS/PAGE and autoradiography in the case of the protein substrate piD261, or by binding to phospho-cellulose paper and scintillation counting¹¹ in the case of the peptide substrate.

Identification of CK2 phosphorylation site in Akt1

For the determination of the *in vitro* phosphorylation site, Akt1 was phosphorylated by CK2 and purified by SDS/PAGE. In the case of *in vivo* phosphorylation, w.t. Akt was overexpressed in 293T cells; before lysis, cells were treated for 2 h with 1 μ M okadaic acid, in order to increase the phosphorylation degree; Akt was purified by immunoprecipitation and SDS/PAGE. In both cases, the gel band corresponding to the protein was excised and the protein digestion was performed in gel according to Gharahdaghi.³⁸ The digested protein was then analyzed by MALDI mass spectrometry using a MALDI-TOF (Micromass) and α -cyano-4-hydroxycinnamic acid as matrix (Sigma). The protein fingerprinting was analyzed with Mascot search engine (Matrix Science, UK) to highlight possible phosphorylated peptides, using Ser/Thr phosphorylation as variable modification. A peptide corresponding to the sequence [122–142] of Akt1 was identified as possibly phosphorylated. The actual phosphorylation was proved chemically dephosphorylating the peptide.^{39,40} The sample was treated with 10 μ l of Ba(OH)₂ for 30 min at room temperature. The reaction was quenched by adding 2 μ l of formic acid and the sample was desalted using ZipTip (Millipore). The digested protein was then analyzed again by MALDI mass spectrometry and the phosphorylation of the peptide [122–142] was proved by a 98 Da shift of the peak, corresponding to the β -elimination of phosphate from Ser or Thr residue. The identification of the exact phosphorylated site was made by acquiring an MS/MS spectrum of the phosphopeptide [122–142] using an Ultima Q-TOF (Micromass). The spectrum was manually interpreted according to Hunt *et al.*⁴¹ and the position of the phosphorylated site was determined.

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