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Phosphorylation of casein kinase 1 delta: Identification of Ser370 as the major phosphorylation site targeted by PKA *in vitro* and *in vivo*

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Short title: CK1 δ is phosphorylated by PKA at Ser370

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Abbreviations used: CK1 : casein kinase 1 delta; CK1 δ CK: casein kinase 1 delta C-terminal targeted protein kinase; PKA: cAMP dependent protein kinase; PKC α : protein kinase C α , CLK2: CDC-like kinase 2, Akt: protein kinase B, GST: glutathione-S-transferase; DMEM: Dulbecco's modified Eagle's medium; FCS: fetal calf serum; AKAP's: A-kinase anchoring proteins; PP2A: protein phosphatase 2A; PP2B: protein phosphatase 2B; H89: [N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinidine sulfonamide, 2HCl Salt]; MCS: multiple cloning site; FP: fusion protein, aa: amino acid, wt: wildtype, mt: mutant

ABSTRACT

The involvement of CK1 δ in the regulation of multiple cellular processes implies a tight regulation of its activity on different levels. At the protein level reversible phosphorylation plays an important role in modulating the activity of CK1 δ . In the present study, we show that PKA, Akt, CLK2 and PKC α phosphorylate CK1 δ . PKA was identified as the major cellular CK1 δ C-terminal targeted protein kinase (CK1 δ CK) activity phosphorylating CK1 δ *in vitro* and *in vivo*, since i) PKA is detectable in the CK1 δ CK kinase peak fraction of fractionated MiaPaCa-2 cell extracts, ii) PKA shares nearly identical kinetic properties to CK1 δ CK and (iii) both, PKA and CK1 δ CK, phosphorylate CK1 δ at Ser370 *in vitro*. Furthermore, phosphorylation of CK1 δ by PKA decreases substrate phosphorylation of CK1 δ *in vitro*. Mutation of Ser370 to alanine increases the affinity of CK1 δ to phosphorylate β -casein and the GST-p53¹⁻⁶⁴ fusion protein *in vitro* and enhances the formation of an ectopic dorsal axis during *Xenopus laevis* development. Anchorage of PKA and CK1 δ to centrosomes is mediated through AKAP450. Interestingly, preincubation of MiaPaCa-2 cells with the synthetic peptide St-Ht31, which prevents the binding between AKAP450 and the regulatory subunit RII of PKA, results in a 6-fold increase of the activity of CK1 δ . In summary, we conclude that PKA phosphorylates CK1 δ predominantly at Ser370 *in vitro* and *in vivo* and that site-specific phosphorylation of CK1 δ by PKA plays an important role in modulating CK1 δ dependent processes.

Key words: CK1 δ , PKA, AKAP's, Akt, CLK2, PKC α

INTRODUCTION

CK1 represents a unique group within the superfamily of serine/threonine specific kinases found in all eukaryotic organisms. So far 7 mammalian CK1 isoforms (α , β , γ_1 , γ_2 , γ_3 , δ and ϵ) and their splice variants have been characterized. They are all highly conserved within their kinase domains, while significantly differing in length and primary structure of their N-terminal and C-terminal regulatory domains (reviewed in [1]). CK1 isoforms can phosphorylate a wide range of substrates bearing either a canonical or a non canonical consensus sequence [2-7]. As a result, they can influence the activity of numerous key regulator proteins. Processes modulated by CK1 include cell cycle progression and cytokinesis [8], DNA repair and recombination processes [9-12], chromosome segregation, microtubule dynamics, kinetochore and centrosome specific functions [13-16] and apoptosis [17-20]. Consequently, the interest in developing inhibitors specifically targeting CK1 family members has significantly increased the last years.

Several mechanisms have been identified that modulate the activity of CK1 *in vitro* and *in vivo*. Alternative splicing represents a mechanism that influences substrate binding, turnover and subcellular localisation of certain CK1 isoforms ([1] and references therein). In addition, interaction with cellular proteins influences the subcellular localisation of CK1 [21-23] and can inhibit its activity [24]. It has been reported that the subcellular localisation of CK1 δ is an important factor for the regulation of its kinase-substrate interactions and depends on the enzymatic activity of the kinase [15]. Furthermore, autophosphorylation provides an additional mechanism to downregulate CK1 activity [25-27]. However, mechanisms exist to overcome the inhibitory effect of autophosphorylation [28]. Other processes that can regulate CK1 activity include; i) stimulation of cells with insulin, ii) viral transformation, iii) treatment of cells with topoisomerase inhibitors, or iv) γ -irradiation, all resulting in elevated CK1 activity ([1] and references therein). Recently, the increase of CK1 ϵ kinase activity by Wnt-induced dephosphorylation of CK1 ϵ autophosphorylation sites, probably by PP2A, was shown *in vivo* [29]. Similarly, in neostriatal neurons, the metabotropic glutamate receptors activate CK1 ϵ by PP2B [30-32].

Differences in the expression and activity levels of CK1 δ have been detected in organs of young adult Balb/C mice [33]. It has been suggested that the observed differences could be due to alterations in the degree of autophosphorylation or to changes in the activity of cellular kinases that are able to phosphorylate CK1 δ . However, the intramolecular autophosphorylation status of CK1 δ makes it difficult to identify such cellular kinases. Therefore, a biochemical approach was taken using GST-CK1 δ fusion proteins as substrates, which do not exhibit any kinase activity and single fractions of fractionated MiaPaCa-2 cell extracts as source of cellular kinases. We identified PKA as the main cellular kinase activity that phosphorylates CK1 δ predominantly at Ser370. In addition, we showed that Akt, CLK2 and PKC α phosphorylate CK1 δ at Ser370 as well as at other sites within amino acids 305 to 375 *in vitro*. Moreover, phosphorylation of CK1 δ by PKA negatively influenced the substrate phosphorylation efficiency of CK1 δ . Mutation of Ser370 to alanine enhanced the ability of CK1 δ to phosphorylate β -casein and GST-p53¹⁻⁶⁴ *in vitro* and increased the formation of an ectopic dorsal axis during *Xenopus* development. Inhibition of the interaction of PKA with AKAP's by the synthetic St-Ht31 inhibitory peptide led to a significant upregulation of the activity of CK1 δ in MiaPaCa-2 cells. Our data suggest that PKA has an important regulatory effect on the activity of CK1 δ .

MATERIALS AND METHODS

Construction of plasmids

Various CK1 δ fragments were amplified by PCR using wt rat CK1 δ (NM 139060, gi 2053176) as a template and the primers listed in Table 1.

The PCR fragments were first cloned into the MCS of pcDNA-3.1/V5-His-TOPO (Invitrogen, Germany) and then subcloned into the NcoI and XhoI sites of pGEX4T-3 generating the GST-CK1 δ fusion proteins shown in Table 2.

The plasmid pGEX2T-CK1 δ ^{1-428/S370A} containing mutations at base pairs 1108 and 1109 (ag→gc) of rat CK1 δ was constructed using the QuikChange site-directed mutagenesis kit according to manufacturers' instructions (Stratagene, USA). The plasmid pGEX2T-CK1 δ ¹⁻⁴²⁸ served as template and the complementary primers used were CK1 δ 20 (5' primer) and CK1 δ 21 (3' primer) (see Tables 1 and 2).

Rat wt CK1 δ was amplified by PCR using pGEX2T-CK1 δ ¹⁻⁴²⁸ (449, [34]) as template and the primers CK1 δ 22 (5' primer) and CK1 δ 23 (3' primer) (see Tables 1 and 2) and cloned into the pcDNA-3.1/V5-His-TOPO vector generating pcDNA-3.1-HA-CK1 δ (1101).

Rat mt CK1 δ S370A was amplified by PCR using pGEX2T-CK1 δ ^{1-428/S370A} (1021) as template and the primers CK1 δ 22 (5' primer) and CK1 δ 23 (3' primer) (see Tables 1 and 2) and cloned into the pcDNA-3.1/V5-His-TOPO vector generating pcDNA-3.1-HA-mtCK1 δ ^{S370A} (1102).

The kinase dead rat CK1 δ (mt CK1 δ K38M) was amplified by PCR using pGBKT7-CK1 δ ^{1-428/K38M} (737, [23]) as template and the primers CK1 δ 24 (5' primer / myc-tag) and CK1 δ 25 (3' primer) (see Tables 1 and 2) and then cloned into the pcDNA-3.1/V5-His-TOPO vector, generating pcDNA-3.1-myc-mtCK1 δ ^{K38M}.

All plasmids were sequenced at GATC Biotech AG (Germany).

Cell lines

Cells (MiaPaCa-2 [35], BxPc3 [36], Colo357 [37] and PancTu1 [38]) were grown in a 1:1 (vol/vol) mixture of DMEM and RPMI medium supplemented with 10% FCS, 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified 5% carbon dioxide atmosphere at 37°C.

Antibodies

The polyclonal antibody against PKA (Upstate, GB) and the monoclonal CK1 δ specific antibody 128A (ICOS Corporation, USA) were used. Horseradish peroxidase conjugated with either anti-rabbit or anti mouse IgG was purchased from GE Healthcare, GB.

Treatment of cells, labelling of cells and cell lysis

MiaPaCa-2 cells were treated with either the PKA specific inhibitor H89 (5 μ M; Calbiochem, Germany), the PKA activator forskolin (20 μ M; Calbiochem, Germany), the St-Ht31 inhibitory peptide (10 μ M; Promega, USA) or the St-Ht31P control peptide (10 μ M; Promega, USA). Subconfluent MiaPaCa-2 cells were labeled with 2 mCi/ml of [³²P]-orthophosphate (GE Healthcare, GB) in Pi-free DMEM supplemented with 5% Pi-free FCS in the presence of either H89 (5 μ M) or forskolin (20 μ M) for 2 hours. Cells were washed in ice cold PBS and lysed either in sucrose lysis buffer (20 mM Tris-acetate pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 4 μ g/ml leupeptin, 30 μ g/ml aprotinin, 0.1% β -mercaptoethanol) or in NP40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 5 mM DTT, 1 mM EDTA, 1 mM EGTA, 50 μ M leupeptin and 30 μ g/ml aprotinin).

Fractionation of cell extracts

MiaPaCa-2, BxPc3, Colo357 and PancTu1 cells were lysed in sucrose lysis buffer. Cell lysates were passed through a 0.40 μ m filter and in each case 3 mg of total protein was applied to an anion exchange column (Resource-Q) attached to an ETTAN purifier (GE Healthcare, GB). The proteins were eluted with a linear ascending gradient between 0-1000 mM NaCl in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 5% glycerol, 0.04% Brij, 1 mM benzamidine, 4 μ g/ml leupeptin and 0.1% β -mercaptoethanol.

Western blot analysis

For the detection of CK1 δ and PKA cells were lysed in NP40 lysis buffer. Extracts were clarified by centrifugation and the protein concentration of the lysates was determined.

75 µg of the protein extract were separated on 12.5% SDS-polyacrylamide gels and transferred onto a nitrocellulose blotting membrane (Hybond C super, GE Healthcare, GB). The membranes were probed with the appropriate antibodies. Immunocomplexes were detected using anti-mouse or anti-rabbit peroxidase conjugated IgG, followed by chemiluminescence detection (ECL; GE Healthcare, GB).

Overproduction and purification of Glutathione S-Transferase fusion proteins

The production and purification of the GST-fusion proteins FP267, FP380, FP449, FP882, FP894, FP897, FP898, FP1001, FP1003, FP1004, FP1006, FP1011, FP1012, FP1013, FP1014, FP1020, FP1021, FP1022, FP1023 were carried out as described elsewhere [23].

***In vitro* kinase assays and phosphopeptide analysis**

In vitro kinase assays were carried out in the absence or presence of specific kinase inhibitors (5 µM H89 (PKA), 200 nM deguelin (Akt), or 1 µM calphostin C (PKC α)) as described previously [34]. Different GST-CK1 δ fusion proteins; GST-p53¹⁻⁶⁴ (FP267), GST-p53^{1-85/S4,6,9A} (FP380), the CK1 peptide substrate (Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg, (Promega, UK), [39] and β -casein (Sigma, Germany) were used as substrates. Recombinant PKA (Calbiochem, Germany), purified PKA catalytic subunit from porcine heart (Sigma, Germany), recombinant human PKC α kinase/aa 1-672 (Biomol, Germany), recombinant human Akt kinase/aa 106_480 (Biomol, Germany), recombinant human CLK2 kinase (Invitrogen, Germany) or single fractions of fractionated MiaPaCa-2 cell extracts were used as sources of enzyme. Phosphorylated proteins were separated by SDS-PAGE and the protein bands were visualised on dried gels by autoradiography. Where indicated, the phosphorylated protein bands were excised and quantified by Cherenkov counting. Phosphopeptide analyses of *in vitro* and *in vivo* labeled proteins were performed as described earlier [23].

RNA synthesis

For microinjection experiments, run-off transcripts of rat wt HA tagged CK1 δ plasmid 1101 , HA tagged mutant CK1 δ S370A (plasmid 1102) and myc tagged kinase dead CK1 δ K38M (plasmid 737) were synthesised using the T7 Message Machine Kit from Ambion.

***Xenopus* microinjection**

Xenopus embryos were microinjected into both ventral blastomeres at 4 cell stage with the indicated amount of RNA (total amount). Secondary axis formation was analysed at stage 22 and stage 26 of development. Staging was according to Nieuwkoop and Faber [40].

RESULTS

CK1 δ activity differs in pancreatic tumour cell lines

Recently, it has been suggested that the differences in the activity of CK1 δ in extracts of various organs of young adult Balb/C mice might be influenced by site specific phosphorylation of CK1 δ [33]. To further follow up this idea the activity of CK1 δ was analysed in fractionated extracts of highly apoptosis-resistant pancreatic tumour cell lines, all expressing similar protein amounts of CK1 δ . The kinase activity in fractionated BxPc3, MiaPaCa2, PancTu1 and Colo357 cell extracts, which eluted at 220-240 mM NaCl and was inhibited by the CK1 specific inhibitor IC261 [41], differed up to six fold (see supplemental material, Figure 1S). These results suggest that post-translational modifications of CK1 δ ,— especially site-specific phosphorylation, are involved in modulating the activity of CK1 δ .

Cellular kinases phosphorylate CK1 δ at Ser370

The C-terminal domain of CK1 δ plays an important role for the regulation of the kinase activity of CK1 δ . In order to identify cellular kinases, which were able to phosphorylate CK1 δ within its C-terminal domain, protein extracts of MiaPaCa-2 cells were prepared and fractionated by ion exchange chromatography using a Resource-Q column. Each eluted fraction was tested for kinase activity using GST-CK1 $\delta^{305-375}$ as substrate. A major kinase peak was detected between 160 and 190 mM NaCl (Figure 1A), indicating that GST-CK1 $\delta^{305-375}$ can be phosphorylated by cellular kinases *in vitro*.

To localise the CK1 δ CK dependent phosphorylation site(s) within the C-terminal region of CK1 δ , additional GST-CK1 δ fusion proteins comprising amino acids 330-375, 305-357 and 353-375 were constructed (Tables 1 and 2) and used as substrates for phosphorylation by the CK1 δ CK activity. While the cellular CK1 δ CK activity (peaking in fraction 10) phosphorylated the truncated GST-CK1 δ fusion proteins GST-CK1 $\delta^{330-375}$ and GST-CK1 $\delta^{353-375}$ to a similar extent (96% and 98%, respectively) compared to GST-CK1 $\delta^{305-375}$ (100%), this activity phosphorylated GST-CK1 $\delta^{305-357}$ to approximately

38 % (Figure 1B). Taken together, these results suggest that the major phosphorylation site(s) targeted by cellular kinases are localised within amino acids 353-375 of CK1 δ .

A Scansite research (<http://scansite.mit.edu>) revealed that within amino acids 353-375 of CK1 δ Ser370 might be the major phosphorylation site targeted by cellular kinases among them Akt, PKA, PKC $\alpha/\beta/\gamma/\zeta$ and CLK2. Therefore, a GST-CK1 $\delta^{353-375/S370A}$ fusion protein bearing a serine to alanine mutation at position 370 was generated and used as substrate in *in vitro* kinase assays. As shown in Figure 1B, the degree of phosphorylation of the GST-CK1 $\delta^{353-375/S370A}$ fusion protein was approximately 4 fold lower compared to that of the GST-CK1 $\delta^{353-375}$ fusion protein. These data strongly suggest that the major phosphorylation site of CK1 δ targeted by the CK1 δ CK activity is Ser370.

To prove the ability of PKA, Akt, PKC α and CLK2 to phosphorylate CK1 δ at Ser370 several GST-CK1 δ fusion proteins (GST-CK1 $\delta^{305-375}$, GST-CK1 $\delta^{353-375}$, GST-CK1 $\delta^{353-375/S370A}$, GST-CK1 $\delta^{305-357}$, GST-CK1 $\delta^{330-375}$, GST-CK1 $\delta^{318-375,S318A/T323A}$, GST-CK1 $\delta^{353-375, T323A/T329A}$ and GST-CK1 $\delta^{331-375,S331A}$) were used in *in vitro* kinase assays. The results presented in Figure 2A indicate that Akt predominantly phosphorylates Ser370 *in vitro*. In contrast, Ser370 is only weakly phosphorylated by PKC α and Clk2 *in vitro*. The major phosphorylation sites targeted by these two kinases are located within aa 305 to aa 353 of CK1 δ (Figure 2A).

Incubation of a series of GST-CK1 δ fusion proteins (GST-CK1 $\delta^{305-375}$, GST-CK1 $\delta^{330-375}$, GST-CK1 $\delta^{305-357}$, GST-CK1 $\delta^{318-351S318A}$, GST-CK1 $\delta^{305-343}$, GST-CK1 $\delta^{341-375}$, GST-CK1 $\delta^{353-375/S370A}$ and GST-CK1 $\delta^{353-375}$) with the catalytic subunit of PKA purified from porcine heart and [γ - 32 P] ATP confirmed that Ser370 is the major phosphorylation site targeted by PKA *in vitro* (Figure 2B).

To prove the stoichiometry of the phosphorylation by PKA *in vitro* kinase assays were performed using purified PKA as enzyme and GST-CK1 $\delta^{305-375}$ as substrate. The data of three independent experiments indicated that approximately 1.5 moles of phosphate were incorporated per mole of protein suggesting that there is one major phosphorylation site within amino acids 305 to 375 being phosphorylated by PKA *in vitro* (Figure 2C).

To examine whether additional serine/threonine residues of CK1 δ are phosphorylated by PKA *in vitro*, the GST-CK1 δ fusion proteins GST-CK1 δ^{1-428} , GST-CK1 δ^{1-375} , GST-CK1 δ^{1-350} , GST-CK1 δ^{52-304} and GST-CK1 $\delta^{149-428}$ were used as substrates. All GST-CK1 δ fusion proteins containing Ser370 (GST-CK1 δ^{1-428} , CK1 $\delta^{149-428}$, GST-CK1 δ^{1-375}) were phosphorylated by PKA. Even though GST-CK1 δ^{1-428} and GST-CK1 δ^{1-375} were highly autophosphorylated, their degree of phosphorylation increased up to 82% in the presence of purified PKA. In contrast, GST-CK1 δ fusion proteins that did not encompass Ser370 (GST-CK1 δ^{1-350} and GST-CK1 δ^{52-304}) were either weakly or not phosphorylated. Interestingly, when the GST-CK1 $\delta^{1-428S370A}$ fusion protein was used as substrate, no changes in the phosphorylation status of CK1 δ were observed underlining that PKA mediated phosphorylation occurs predominantly at Ser370 *in vitro* (Figure 2D).

To gain more information regarding the identity of the CK1 δ CK activity, the CK1 δ CK activity was assayed in the absence and presence of kinase inhibitors specific for PKA, PKC α or Akt using GST-CK1 $\delta^{305-375}$ as substrate. As presented in Figure 3A, a 65% reduction of the CK1 δ CK activity was observed in the presence of the PKA specific inhibitor H89 (5 μ M), whereas in the presence of the PKC specific inhibitor, calphostin C, (1 μ M) or the Akt specific inhibitor, degeulin (200 nM),- the reduction of the CK1 δ CK activity was much lower (25% and 9%, respectively).

As PKA seems to be the major CK1 δ CK activity present in the kinase peak fractions (Figure 1A) and as PKA could be clearly detected in the kinase peak fractions 10 and 11 by Western blot analysis (Figure 3B), the kinetic parameters of the CK1 δ CK activity were compared with those of recombinant PKA using the GST-CK1 $\delta^{353-375}$ fusion protein as substrate. The results indicate that the K_m and V_{max} values for CK1 δ CK and PKA are nearly identical (Figure 3C). Taken together, all these experiments strongly point to PKA being the principal kinase responsible for the phosphorylation of CK1 δ at Ser370.

Ser370 plays an important role in modulating the substrate phosphorylation of CK1 δ *in vitro*

In order to examine the importance of Ser370 for the catalytic activity of CK1 δ *in vitro*, the kinetic constants of GST-CK1 δ^{1-428} and GST-CK1 $\delta^{1-428S370A}$ mediated

phosphorylation of β -casein and GST-p53¹⁻⁶⁴ were experimentally calculated. As presented in Figure 2S (see supplemental material) GST-CK1 δ ^{1-428S370A} has lower K_m and higher V_{max} values compared to those of GST-CK1 δ ¹⁻⁴²⁸. These observed differences revealed that CK1 δ ^{1-428S370A} has a higher affinity to phosphorylate β -casein and GST-p53¹⁻⁶⁴ compared to that of GST-CK1 δ ¹⁻⁴²⁸, which is furthermore indicated by the efficiency parameter (V_{max}/K_m ratio) (Figure 2S, C).

Phosphorylation of GST-CK1 δ by PKA affects its activity *in vitro*

So far, our results clearly indicate that S370, which plays an important role in the modulation of the activity of CK1 δ , is the major phosphorylation site targeted by PKA *in vitro*. To examine the influence of PKA phosphorylation on the activity of CK1 δ , *in vitro* kinase assays were performed in the presence or absence of the catalytic subunit of PKA purified from porcine heart. GST-CK1 δ ¹⁻⁴²⁸ (FP 449) was used as source of enzyme activity. CK1 δ was preincubated with [γ -³²P] ATP for 15 min before adding GST-p53¹⁻⁶⁴ (FP267) as substrate either alone or in combination with PKA for the indicated time points (see supplemental material, Figure 3S).

As presented in Figure 3S, CK1 δ mediated phosphorylation of GST-p53¹⁻⁶⁴ (FP267) was reduced by up to 80% in the presence of PKA compared to that observed in the absence of the catalytic subunit of PKA. Phosphorylation of GST-p53¹⁻⁶⁴ by PKA could be excluded since *in vitro* kinase assays demonstrated that PKA does not phosphorylate GST-p53¹⁻⁶⁴ (data not shown).

Effects of CK1 δ S370A in *Xenopus* embryogenesis

Different CK1 isoforms have been implicated in the Wnt signaling pathway. Considering the high affinity of CK1 δ S370A to phosphorylate β -casein and GS-p53¹⁻⁶⁴ *in vitro* we now analysed the effects of this mutant on WNT/ β -catenin signaling *in vivo* using the *Xenopus* secondary axis induction assay.

In vitro transcribed RNA encoding either HA-tagged rat wt CK1 δ , mt CK1 δ S370A or myc-tagged mt CK1 δ K38M were microinjected into both ventral cells of 4 cell-stage *Xenopus* embryos. The kinase dead mutant CK1 δ K38M did not effect embryonic development whereas high doses (2 ng) of wtCK1 δ or CK1 δ S370A caused the formation

of an ectopic dorsal axis (Figure 4 A, B) as well as gastrulation movements defects. To identify any difference in biological activity of both constructs, we lowered the amount of injected RNA in subsequent experiments and selected for experiments with a low gastrulation phenotype (i.e. not more than 10% as this can occur non-specifically in egg batches of low quality or eventually after injection of higher RNA amounts). At lower amounts of microinjected mRNA the secondary axis phenotype was seen more often in embryos expressing exogenous HA-tagged CK1 δ S370A than in embryos expressing wt CK1 δ (Figure 4 C).

PKA phosphorylates CK1 δ *in vivo*

To examine whether CK1 δ is phosphorylated by PKA in tissue culture cells, experiments were performed on the basis that modulating the activity of PKA should influence the phosphorylation status of CK1 δ . For this purpose, MiaPaCa-2 cells were treated either with the PKA specific inhibitor H89 (5 μ M) or with the PKA activator forskolin (20 μ M) in the presence of 2 mCi [³²P]-orthophosphate for two hours. Cells were lysed and phosphorylated CK1 δ was immunoprecipitated, isolated, digested with trypsin and oxidised. The resulting tryptic phosphopeptides were separated by two dimensional tryptic phosphopeptide mapping and compared with the phosphopeptides derived from the GST-CK1 δ ³⁰⁵⁻³⁷⁵ fusion protein, which was phosphorylated by PKA *in vitro*.

Phosphopeptides 1 and 2 were present in CK1 δ isolated from MiaPaCa-2 cells treated with forskolin (Figure 5A) and in GST-CK1 δ ³⁰⁵⁻³⁷⁵ (Figure 5C), whereas these peptides were not detectable in CK1 δ isolated from MiaPaCa-2 cells treated with H89 (Figure 5B) pointing to PKA as the cellular kinase responsible for the phosphorylation of amino acid residues within these two peptides. Other migrated peptides (labeled 3 and 4) could be detected in the phosphopeptide analyses of CK1 δ isolated from both, forskolin and H89 treated cells indicating the existence of additional phosphorylation sites.

The subcellular localisation of PKA is important for modulating the activity of CK1 δ

Compartmentation of signal transduction molecules (e.g. kinases and phosphatases) occurs by anchoring to scaffold proteins. This then allows substrate

phosphorylation/dephosphorylation in concert with various kinases and phosphatases resulting in the regulation of the functions of the substrate, specific for a certain compartment.

Recently, it has been reported that interaction of PKA and CK1 δ with AKAP450 docks both kinases to centrosomes [22, 42, 43] along with other signal transduction molecules. The binding of PKA to AKAP's can be blocked by a membrane-permeable AKAP St-Ht31 inhibitory peptide [42, 44]. Therefore, we investigated whether disruption of the PKA-AKAP complex by the anchoring disrupting peptide St-Ht31 [45, 46] can influence the activity levels of CK1 δ . For this purpose, MiaPaCa-2 cells were treated with the St-Ht31 specific inhibitory peptide (10 μ M) or the St-Ht31P control peptide [47] for 3 h. Cell lysates were fractionated by anion exchange chromatography and the CK1 δ kinase activity was determined. When the CK1 substrate peptide (Figure 6B) or GST-p53¹⁻⁶⁴ (FP267) (Figure 6A) were used as substrates, the CK1 δ activity increased 3 to 6 fold in cells treated with the St-Ht31 inhibitory peptide compared to untreated cells and cells treated with the St-Ht31P control peptide (Figure 6).

Respectively, differences in the CK1 δ kinase activity were observed in cells treated with the St-Ht31 inhibitory peptide when GST-p53^{1-85/S4, 6, 9A} (FP380) was used as substrate, compared to untreated cells and cells treated with the St-Ht31P control peptide (Figure 6A). However, as expected, the CK1 δ mediated phosphorylation of FP380 was significantly lower compared to that of FP267 due to the mutation of most of the CK1 δ phosphorylation sites (S4, 6, 9A) within the N-terminal domain of p53.

DISCUSSION

CK1 δ , a member of the CK1 family, is involved in the regulation of many different cellular processes including cell proliferation and cell death. Therefore, several mechanisms ensure a tight regulation of CK1 δ on different levels [15, 24, 26, 28, 48, 49].

Using a biochemical approach to clarify the role of cellular kinases in modulating the activity of CK1 δ , a CK1 δ CK activity eluting between 160-190 mM NaCl was detected in fractionated MiaPaCa-2 cell lysates. The use of various C-terminal truncated wt and mt GST-CK1 δ fusion proteins as substrates revealed that mainly Ser370 is targeted by CK1 δ CK. Ser370 is a potential phosphoacceptor site for phosphorylation by several putative kinases among them Akt, PKA, CLK2 and PKC α . *In vitro* kinase assays using various GST-CK1 δ fusion proteins as substrates revealed that PKA and Akt mainly phosphorylate Ser370, while the key targeted sites of PKC α and CLK2 lie within aa 305-353 of CK1 δ . However, additional experiments are necessary to identify the phosphorylation sites targeted by PKC α and CLK2 and to elucidate their role in regulating the activity of CK1 δ .

Focussing on the phosphorylation of Ser370, our data strongly suggest that this serine residue is predominantly phosphorylated by PKA, since (i) PKA co-eluted with the peak of the CK1 δ CK activity as verified by Western blot analysis, (ii) the PKA specific inhibitor H89 inhibited the kinase activity present in the kinase peak fraction up to 65% whereas in the presence of the Akt specific inhibitor degeulin or in the presence of the PKC specific inhibitor calphostin C a much weaker reduction was observed, (iii) the comparison of the kinetic parameters of recombinant PKA with those of CK1 δ CK present in the kinase peak fraction indicated nearly identical values and (iv) the purified catalytic subunit of PKA, as well as recombinant PKA, phosphorylated all GST-CK1 δ fusion proteins containing Ser370 *in vitro*, whereas all GST-CK1 δ fusion proteins lacking Ser370 or containing a serine to alanine substitution at Ser370 were not phosphorylated. Furthermore, time course experiments revealed that there is one major phosphorylation site for PKA within amino acids 305-375. The presence of arginine and lysine residues at positions n-3 and n-2 on the N-terminal side of Ser370 (R265ERKVS370) resembles a perfect consensus recognition motif for PKA [50] thereby favouring Ser370 as the main phosphorylation site targeted by PKA. Moreover, comparison of our

phosphopeptide analyses suggests that CK1 δ is phosphorylated *in vivo* at the same sites being phosphorylated by PKA *in vitro*.

In addition, phosphorylation of CK1 δ by PKA reduced its ability to phosphorylate GST-p53¹⁻⁶⁴ up to 80%. This result indicates that site specific phosphorylation is implicated in modulating the activity of CK1 δ .

The importance of Ser370 for the substrate phosphorylation of CK1 δ was indicated by comparing the kinetic parameters of GST-CK1 δ ¹⁻⁴²⁸ with those of GST-CK1 δ ^{1-428S370A}. Our results show that the GST-CK1 δ ^{1-428S370A} mutant exhibits lower K_m and higher V_{max} values compared to those of GST-CK1 δ ¹⁻⁴²⁸ when β -casein and GST-p53¹⁻⁶⁴ were used as substrates.

Furthermore, the role of Ser370 appears to be physiologically important since it has been shown that mutation of this site can interfere with normal development of *Xenopus* embryos. Expression of the mutant kinase (CK1 δ S370A) in *Xenopus* embryos resulted in a higher rate of ectopic dorsal axis formation compared to the expression of wtCK1 δ whereas the kinase dead mutant (CK1 δ K38M) failed in the axis induction assay. In addition, our data strongly support the idea that CK1 δ and PKA interact physiologically based on the observation that inhibition of the interaction of PKA with AKAP's by the St-Ht31 inhibitory peptide [45, 51] resulted in an increase of CK1 δ activity. The increased activity of CK1 δ could be explained by changes in the phosphorylation status of CK1 δ . Loss of the interaction between CK1 δ and PKA could lead to a reduced phosphorylation of Ser370. Furthermore, cellular phosphatases associated with AKAP's [52, 53] could more efficiently dephosphorylate the autophosphorylation sites of CK1 δ in the absence of PKA thereby activating CK1 δ . However, St-Ht31 treatment of cells can also decrease the activity of signalling molecules as it has been shown that St-Ht31 could disrupt the activity of RhoA [54].

In summary, these results show for the first time that CK1 δ is phosphorylated by several kinases and that site specific phosphorylation, especially at Ser370 by PKA, plays an important role in modulating the activity of CK1 δ .

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TABLES AND FIGURES

Table 1 Sequences of CK1 δ -specific primers

Table 2 Generation of CK1 δ -specific fragments and nomenclature of pGEX-CK1 δ plasmids and fusion proteins

Figure 1 Characterisation of CK1 δ CK activities in MiaPaCa-2 cells

(A) Fractionation of CK1 δ CK activities present in MiaPaCa-2 cells by anion exchange chromatography. A soluble protein extract (3 mg) derived from the lysis of MiaPaCa-2 cells was fractionated on a Resouce Q column with a linear ascending gradient of NaCl. Kinase assays were performed using equal amounts of GST-CK1 $\delta^{305-375}$ as substrate (see Coomassie gel) and single fractions of fractionated MiaPaCa2 cell extracts as source of enzyme. Proteins were separated by SDS-PAGE and the phosphorylated proteins were detected by autoradiography. Quantification of phosphate incorporation of phosphorylated GST-p53¹⁻⁶⁴ was measured by Cerenkov counting. A major kinase activity phosphorylating GST-CK1 $\delta^{305-375}$ (FP1006) eluted between 160-190 mM NaCl (fractions 9-11). **(B)** Localisation of phosphorylation sites targeted by CK1 δ CK activities. *In vitro* kinase assays were performed using fractions 7-12 of fractionated MiaPaCa-2 cell extracts as source of enzyme activity and fusion proteins GST-CK1 $\delta^{305-375}$ (FP1006), GST-CK1 $\delta^{330-375}$ (FP1011), GST-CK1 $\delta^{305-357}$ (FP1012), GST-CK1 $\delta^{353-375}$ (FP1022) and GST-CK1 $\delta^{305-375S370A}$ (FP1021) as substrates. The highest cellular kinase activity (100%) was detected in fraction 10 when using the GST-CK1 $\delta^{305-375}$ fusion protein (FP 1006) as substrate. Open squares: GST-CK1 $\delta^{305-375}$, open circles: CK1 $\delta^{330-375}$, closed triangles: CK1 $\delta^{353-375}$, star: CK1 $\delta^{353-375S370A}$, closed circle: CK1 $\delta^{305-357}$, solid line: NaCl concentration.

Figure 2 Phosphorylation of CK1 δ by cellular kinases *in vitro*

(A) Phosphorylation of CK1 δ by Akt, PKC α and CLK2

In vitro kinases assays were performed using the recombinant human PKC α kinase/aa 1-672, the recombinant human Akt kinase /aa106-480 and the recombinant human CLK2 kinase as sources of enzyme activity and various GST-CK1 δ fusion proteins (GST-CK1 δ ³⁰⁵⁻³⁷⁵, GST-CK1 δ ³⁵³⁻³⁷⁵, GST-CK1 δ ^{353-375/S370A}, GST-CK1 δ ³⁰⁵⁻³⁵⁷, GST-CK1 δ ³³⁰⁻³⁷⁵, GST-CK1 δ ^{318-375,S318A/T323A}, GST-CK1 δ ^{353-375, T323A/T329A} and GST-CK1 δ ^{331-375,S331A}) or GST as substrates. Quantification of phosphate incorporation of phosphorylated GST-CK1 δ fusion proteins and GST was measured by Cerenkov counting.

(B) Characterisation of the site specific phosphorylation of CK1 δ by PKA

In vitro kinases assays were performed using the catalytic subunit of PKA purified from porcine heart as enzyme and GST-CK1 δ ³⁰⁵⁻³⁷⁵ (FP 1006), GST-CK1 δ ³³⁰⁻³⁷⁵ (FP1011), GST-CK1 δ ³⁰⁵⁻³⁵⁷ (FP1012), GST-CK1 δ ^{318-351/S318A} (FP1013), GST-CK1 δ ³⁰⁵⁻³⁴³ (FP1014), GST-CK1 δ ³⁴¹⁻³⁷⁵ (FP1020), GST-CK1 δ ^{353-375/S370A} (FP1021) and GST-CK1 δ ³⁵³⁻³⁷⁵ (FP1022) as substrates. Equal amounts of GST-CK1 δ fusion proteins were separated on 12.5% SDS-polyacrylamide gels and stained with Coomassie. Quantification of phosphate incorporation of phosphorylated GST-CK1 δ fusion proteins was measured by Cerenkov counting.

(C) PKA phosphorylates one major residue in the C-terminal domain of CK1 δ . GST-CK1 δ ³⁰⁵⁻³⁷⁵ (FP1006) was phosphorylated *in vitro* by PKA for the indicated times. Up to 1.5 mol phosphate/mol protein was incorporated into the GST-CK1 δ ³⁰⁵⁻³⁷⁵ fusion protein.

(D) Phosphorylation of GST-CK1 δ fusion proteins by PKA. *In vitro* kinases assays were performed using the catalytic subunit of PKA purified from porcine heart as enzyme and GST-CK1 δ ¹⁻⁴²⁸ (FP449), GST-CK1 δ ¹⁴⁹⁻⁴²⁸ (FP894), GST-CK1 δ ^{1-428S370A} (FP1023), GST-CK1 δ ⁵²⁻³⁰⁴ (FP882), GST-CK1 δ ¹⁻³⁵⁰ (FP898) and GST-CK1 δ ¹⁻³⁷⁵ (FP897) as substrates. The autoradiogram, the Coomassie gel as well as the quantification of a representative experiment are presented. Open bars without PKA, grey bars in the presence of PKA.

Figure 3 PKA is the major CK1 δ CK activity present in the kinase peak fraction of fractionated MiaPaCa-2 cells

(A) CK1 δ targeting kinase activities are mainly inhibited by the PKA specific inhibitor H89. *In vitro* kinase assays were performed in the absence and presence of the PKA specific inhibitor H89 (5 μ M), the Akt specific inhibitor degeulin (200 nM) or the PKC specific inhibitor calphostin C (1 μ M) using GST-CK1 $\delta^{305-375}$ (FP1006) as substrate and fractions 5-14 of fractionated MiaPaCa-2 cell lysates as sources of kinase activity. Closed bars: control, hatched bars: H89, grey bars: degeulin; open bars: calphostin C.

(B) PKA is present in the kinase peak fractions. PKA can be detected in the kinase peak fractions 10 and 11 of fractionated MiaPaCa-2 cell lysates in Western Blot analysis.

(C) The kinetic properties of CK1 δ CK and PKA are nearly identical. To determine the kinetic parameters of CK1 δ CK and PKA *in vitro* kinase assays were performed using GST-CK1 $\delta^{353-375}$ (FP1022) as a substrate. The results indicate that the K_m and V_{max} values of CK1 δ CK (closed dots) and PKA (crucifix) are nearly identical.

Figure 4 Secondary axis induction in *Xenopus laevis* embryos.

Xenopus laevis embryos were injected with wt or mt CK1 δ RNA at 4-cell stage as indicated. Evaluation of secondary axis formation was performed at stage 22 or 26. Different views of embryos are shown **(A)**. Quantification of experiments shows that injection of higher amounts results in secondary axis formation and gastrulation movements defects in case of wt CK1 δ and CK1 δ S370A. The kinase dead mutant CK1 δ K38M is inactive in this assay **(B)**. Injection of lower amounts and selection of embryo batches with low gastrulation movement phenotype indicates that CK1 δ S370A has a higher capacity to induce secondary axis in comparison to wt CK1 δ **(C)**.

Figure 5 Phosphopeptide analyses of CK1 δ and GST-CK1 $\delta^{305-375}$

GST-CK1 $\delta^{305-375}$ and [32 P]-labeled CK1 δ isolated from MiaPaCa-2 cells treated either with 20 μ M forskolin **(A)** or with 5 μ M H89 **(B)** for 2 h were oxidised and digested with

trypsin as described in Materials and Methods. In each case the tryptic phosphopeptides were analysed by two dimensional phosphopeptide mapping. The phosphopeptides are numbered from 1 to 6. Arrows indicate those peptides being identical in the *in vitro* and the *in vivo* maps. **(A)** Shows CK1 δ specific phosphopeptides of forskolin treated MiaPaCa-2 cells, **(B)** phosphopeptides of CK1 δ expressed in H89 treated MiaPaCa-2 cells and **(C)** phosphopeptides of the GST-CK1 δ ³⁰⁵⁻³⁷⁵ fusion protein phosphorylated by PKA *in vitro*. An asterisk indicates the loading point.

Figure 6 Inhibition of the interaction of PKA with AKAP's is accompanied by an increased CK1 δ activity

Equal protein amounts (3 mg) of lysates derived from MiaPaCa-2 cells, untreated, treated with 10 μ M of the St-Ht31 control peptide or with 10 μ M of the St-Ht31 specific inhibitory peptide were fractionated as described in Material and Methods. The proteins were eluted with a linear gradient of increasing NaCl concentrations (represented as a solid diagonal line). **(A)** The CK1 δ specific kinase activity was determined using the GST-p53¹⁻⁶⁴ (FP267) and the GST-p53^{1-84 S4, 6, 9A} fusion proteins (FP380) as substrates. The kinase activity is shown as i) open circles: in cells treated with the St-Ht31 inhibitory peptide, ii) open rombs: in cells treated with the St-Ht31 control and iii) closed triangles: in untreated cells when FP267 was used as substrate

The kinase activity is shown as i) closed circles: in cells treated with the St-Ht31 inhibitory peptide, ii) closed rombs: in cells treated with the St-Ht31 control and iii) open triangles: in untreated cells, when FP380 was used as substrate.

(B) The CK1 δ specific kinase activity was also determined using the CK1 peptide substrate Asp- Asp- Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg. Open circles: kinase activity in cells treated with the St-Ht31 inhibitory peptide, open rombs: kinase activity in cells treated with the St-Ht31 control peptide and closed triangles: kinase activity in untreated cells.

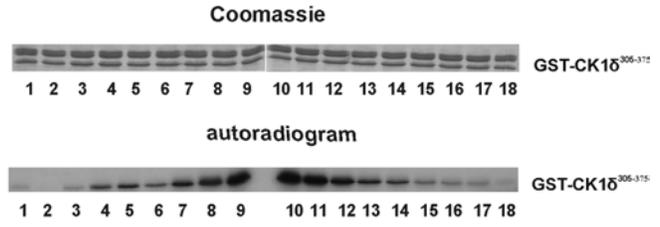
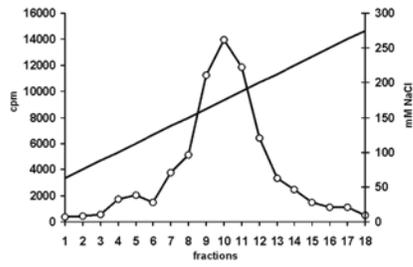
Table 1 Sequences of CK1 δ -specific primers

primer	sequence
CK1 δ -1	5'-CGAATTCCATGGAGAGCAAGATCTACAAAATGAT-3'
CK1 δ -2	5'-ACTCGAGTCGACAGCATCATCTGCAGCC-3'
CK1 δ -3	5'-CGAATTCCATGGACTTTGGGCTGGCCAAG-3'
CK1 δ -4	5'-ACTCGAGTCGACGTAGGTGGTACGTCGTG-3'
CK1 δ -5	5'-CGAATTCCATGGAGCTGAGGGTCGGGAAT-3'
CK1 δ -6	5'-ACTCGAGTCGACTCAACGGTGCAGCCGCA-3'
CK1 δ -7	5'-ACTCGAGTCGACTGAGGTAGGGGTAAGG-3'
CK1 δ -8	5'-CGAATTCCATGGAGCGGGAACGCCG-3'
CK1 δ -9	5'-CCATGGCTTCCGGCCGTCTGCGGGGAACC-3'
CK1 δ -10	5'-ACTCGAGTCGACTCAAGGAGAGGTGTTGGC-3'
CK1 δ -11	5'-CCATGG <u>CC</u> CGGAATCCAGCCACTCGTGGC-3'
CK1 δ -12	5'-ACTCGAGTCGACTCAATGTGAGGTAGGGGT-3'
CK1 δ -13	5'-ACTCGAGTCGACTCATGGGGGAGCCACTTC-3'
CK1 δ -14	5'-CCATGGCTCCCCAACGCCCTTACCCCT-3'
CK1 δ -15	5'-CCATGGCCAACACCTCTCCTAGACCCGTC-3'
CK1 δ -16	5'-ACTCGAGTCGACTCAACGGTGCAGCCGCATAG <u>CC</u> CAC-3'
CK1 δ -17	5'-CCATGG <u>CC</u> CGGAATCCAGCC <u>G</u> CTCGTGGC-3'
CK1 δ -18	5'-CCATGG <u>G</u> CTCGTGGCCTCCCTTCTGCAGCT-3'
CK1 δ -19	5'-CCATGG <u>CC</u> CGGCCGTCTGCGGGGAACCCAG-3'
CK1 δ -20	5'-CGAGAACGGAAAGTGG <u>C</u> TATGCGGCTGCACCGTGGG-3'
CK1 δ -21	5'-GCTCTTGCCTTTCAC <u>CC</u> GATACGCCGACGTGGCACCC-3'
CK1 δ -22	5'-GATATCATGTACCCATACGATGTTCCAGATTACGCTC TTCATATGGCGATGGAGCTGAGG-3'
CK1 δ -23	5'-AGATCTTCAGTAGGTGGTACG-3'
CK1 δ -24	5'-GGTACCATGGAGGAGCAGAAGCTG-3'
CK1 δ -25	5'-GTCGACTCAGTAGGTGGTACGTCGTGG-3'

Table 2 **Generation of CK1 δ -specific fragments and nomenclature of pGEX-CK1 δ plasmids and fusion proteins**

Primer pair	bp of CK1δ	pGEX vector name	GST-CK1δ fusion protein
CK1 δ 1 + CK1 δ 2	154-912	pGEX4T-3-CK1 δ ⁵²⁻³⁰⁴	GST-CK1 δ ⁵²⁻³⁰⁴ (FP882)
CK1 δ 3 + CK1 δ 4	445-1284	pGEX4T-3-CK1 δ ¹⁴⁹⁻⁴²⁸	GSTCK1 δ ¹⁴⁹⁻⁴²⁸ (FP894)
CK1 δ 5 + CK1 δ 6	1-1125	pGEX4T-3-CK1 δ ¹⁻³⁷⁵	GST-CK1 δ ¹⁻³⁷⁵ (FP897)
CK1 δ 5 + CK1 δ 7	1-1050	pGEX4T-3-CK1 δ ¹⁻³⁵⁰	GSTCK1 δ ¹⁻³⁵⁰ (FP898)
CK1 δ 17 + CK1 δ 6	952-1125	pGEX4T-3-CK1 δ ^{318-375/S318A, T323A}	GST-CK1 δ ^{318-375/S318A, T323A} (FP1001)
CK1 δ 18 + CK1 δ 6	967-1125	pGEX4T-3-CK1 δ ^{323-375/T323A, T329A}	GST-CK1 δ ^{323-375/T323A, T329A} (FP1003)
CK1 δ 19 + CK1 δ 6	991-1125	pGEX4T-3-CK1 δ ^{331-375/S331A}	GST-CK1 δ ^{331-375/ S331A} (FP1004)
CK1 δ 8 + CK1 δ 6	913-1125	pGEX4T-3-CK1 δ ³⁰⁵⁻³⁷⁵	GST-CK1 δ ³⁰⁵⁻³⁷⁵ (FP1006)
CK1 δ 9 + CK1 δ 6	988-1125	pGEX4T-3-CK1 δ ³³⁰⁻³⁷⁵	GST-CK1 δ ³³⁰⁻³⁷⁵ (FP1011)
CK1 δ 8 + CK1 δ 10	913-1071	pGEX4T-3-CK1 δ ³⁰⁵⁻³⁵⁷	GST-CK1 δ ³⁰⁵⁻³⁵⁷ (FP1012)
CK1 δ 11 + CK1 δ 12	952-1053	pGEX4T-3-CK1 δ ^{318-351/S318A}	GST-CK1 δ ^{318-351/S318A} (FP1013)
CK1 δ 8 + CK1 δ 13	913-1029	pGEX4T-3-CK1 δ ³⁰⁵⁻³⁴³	GST-CK1 δ ³⁰⁵⁻³⁴³ (FP1014)
CK1 δ 14 + CK1 δ 6	1021-1125	pGEX4T-3-CK1 δ ³⁴¹⁻³⁷⁵	GST- K1 δ ³⁴¹⁻³⁷⁵ (FP1020)
CK1 δ 15 + CK1 δ 16	1057-1125	pGEX4T-3-CK1 δ ^{353-375/S370A}	GST-CK1 δ ^{353-375/S370A} (FP1021)
CK1 δ 15 + CK1 δ 6	1057-1125	pGEX4T-3-CK1 δ ³⁵³⁻³⁷⁵	GST-CK1 δ ³⁵³⁻³⁷⁵ (FP1022)
CK1 δ 20 +CK1 δ 21	1-1284	pGEX2T-CK1 δ ^{1-428S370A}	GST- CK1 δ ^{1-428S370A} (FP1023)

A



B

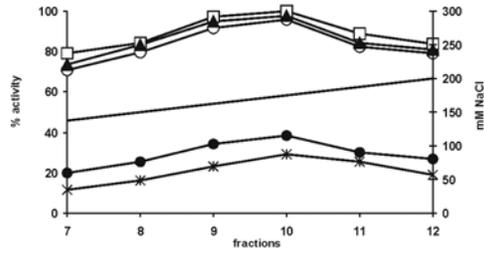


Figure 1

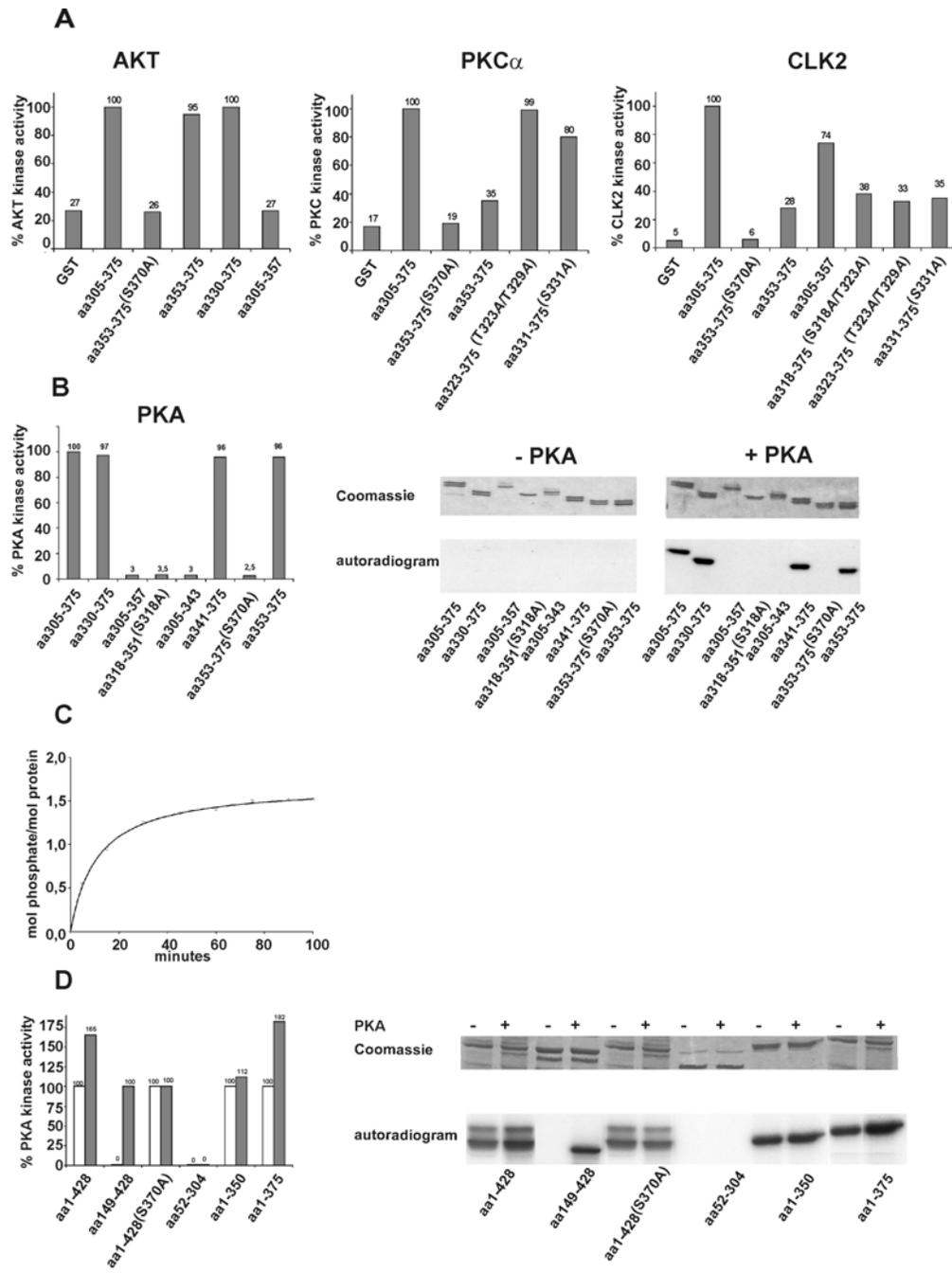
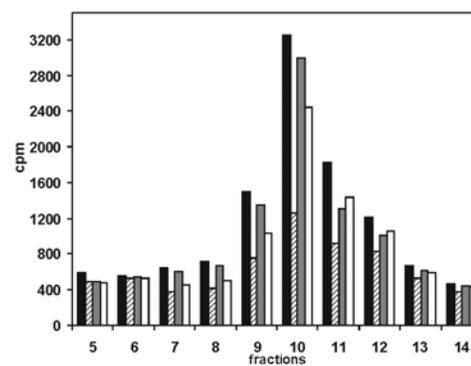
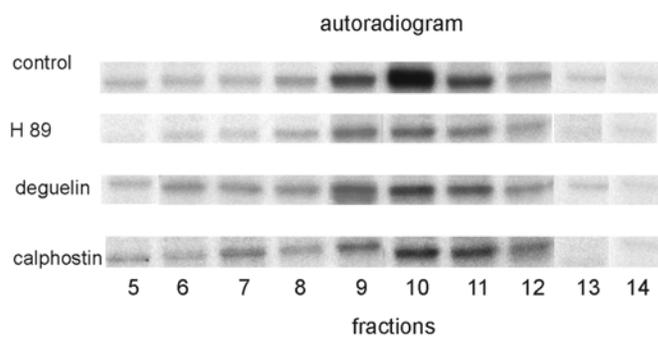
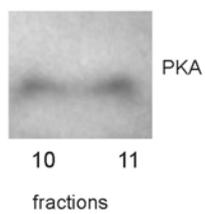


Figure 2

A



B



C

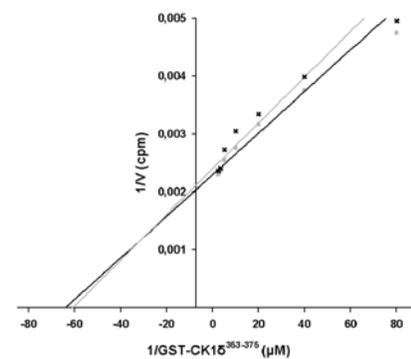
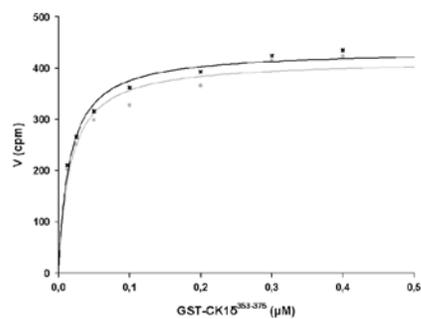
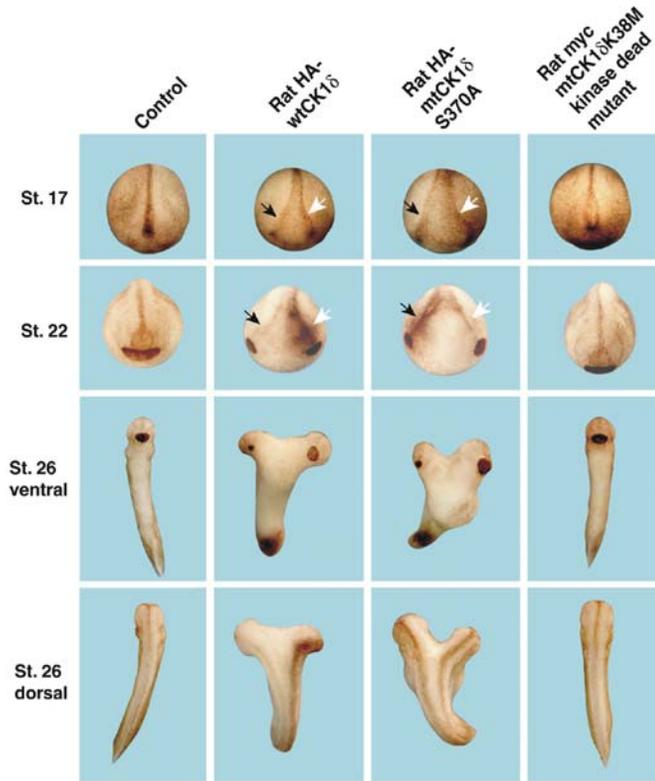
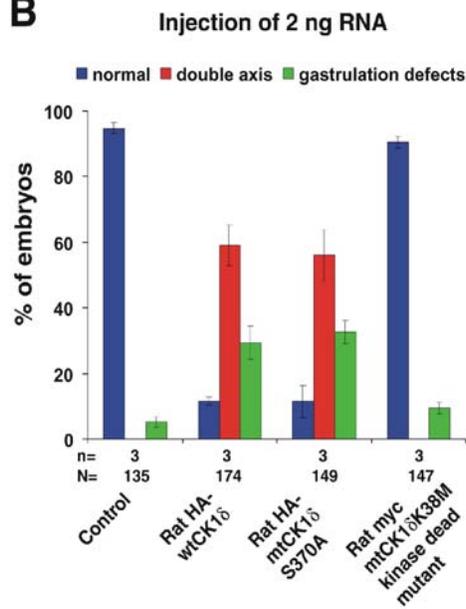


Figure 3

A



B



C

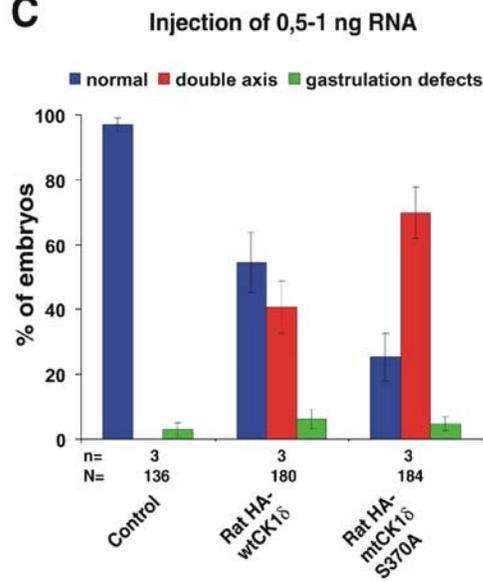


Figure 4

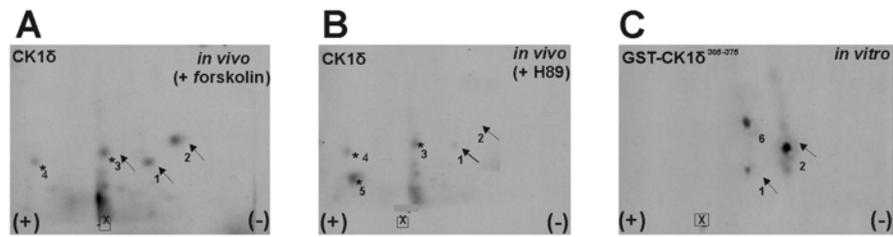


Figure 5

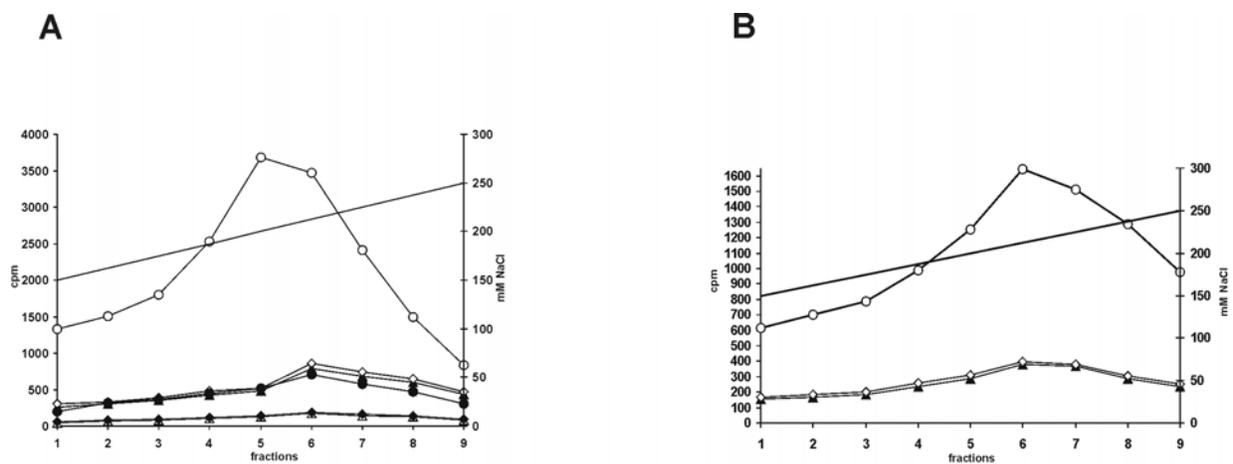


Figure 6