



Phosphorylation of CK1 δ : identification of Ser³⁷⁰ as the major phosphorylation site targeted by PKA *in vitro* and *in vivo*

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The involvement of CK1 (casein kinase 1) δ in the regulation of multiple cellular processes implies a tight regulation of its activity on many 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 (cAMP-dependent protein kinase), Akt (protein kinase B), CLK2 (CDC-like kinase 2) and PKC (protein kinase C) α all phosphorylate CK1 δ . PKA was identified as the major cellular CK1 δ CK (CK1 δ C-terminal-targeted protein kinase) for the phosphorylation of CK1 δ *in vitro* and *in vivo*. This was implied by the following evidence: PKA was detectable in the CK1 δ CK peak fraction of fractionated MiaPaCa-2 cell extracts, PKA shared nearly identical kinetic properties with those of CK1 δ CK, and both PKA and CK1 δ CK phosphorylated CK1 δ at Ser³⁷⁰ *in vitro*. Furthermore, phosphorylation of CK1 δ by PKA decreased substrate phosphorylation of CK1 δ *in vitro*. Mutation of Ser³⁷⁰ to alanine increased the phosphorylation affinity of CK1 δ for β -casein and the GST (glutha-

tion S-transferase)-p53 1–64 fusion protein *in vitro* and enhanced the formation of an ectopic dorsal axis during *Xenopus laevis* development. Anchoring of PKA and CK1 δ to centrosomes was mediated by AKAP (A-kinase-anchoring protein) 450. Interestingly, pre-incubation of MiaPaCa-2 cells with the synthetic peptide St-Ht31, which prevents binding between AKAP450 and the regulatory subunit RII of PKA, resulted in a 6-fold increase in 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: A-kinase-anchoring protein (AKAP), cAMP-dependent protein kinase (PKA), casein kinase 1 δ (CK1 δ), CDC-like kinase 2 (CLK2), protein kinase B (Akt), protein kinase C α (PKC α).

INTRODUCTION

The CK1 (casein kinase 1) group is unique within the superfamily of serine/threonine-specific kinases found in all eukaryotic organisms. So far, seven 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, but differ significantly 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 regulatory proteins. Processes modulated by CK1 include cell-cycle progression and cytokinesis [8], DNA repair and recombination [9–12], chromosome segregation, microtubule dynamics, kinetochore- and centrosome-specific functions [13–16] and apoptosis [17–20]. Consequently, the interest in developing inhibitors to specifically target CK1 family members has increased significantly in recent 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 localization of certain CK1 isoforms [1]. In addition, interaction with cellular proteins influences the subcellular localization of CK1 [21–23] and can inhibit its activity [24]. It has been reported that the subcellular localization 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 down-regulate CK1 activity [25–27]. However, mechanisms exist to overcome the inhibitory effect of autophosphorylation [28]. Other processes that can regulate CK1 activity include stimulation of cells with insulin, viral transformation, treatment of cells with topoisomerase inhibitors or with γ -irradiation, all resulting in elevated CK1 activity [1]. The increase of CK1 ϵ kinase activity by Wnt-induced dephosphorylation of CK1 ϵ autophosphorylation sites, probably performed by PP (protein phosphatase) 2A, 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 differences observed could be the result of alterations in the degree of autophosphorylation or to changes in the activity of cellular kinases which are able to phosphorylate CK1 δ . However, the intramolecular autophosphorylation status of CK1 δ makes it difficult to identify such cellular kinases. In the present study, GST (glutathione S-transferase)-tagged CK1 δ fusion proteins were used as substrates because they do not exhibit any kinase activity, and single fractions of fractionated MiaPaCa-2 cell extracts were used as the source of cellular kinases. We have identified PKA (cAMP-dependent

Abbreviations used: AKAP, A-kinase-anchoring protein; CK1, casein kinase 1; CK1 δ CK, CK1 δ C-terminal-targeted protein kinase; CLK2, CDC-like kinase 2; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GST, glutathione S-transferase; HA, haemagglutinin; HRP, horseradish peroxidase; NP40, Nonidet P40; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PP, protein phosphatase.

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Table 1 Sequences of CK1 δ -specific primers

Primer	Sequence
CK1 δ 1	5'-CGAATTCATGGAGAGCAAGATCTACAAAATGAT-3'
CK1 δ 2	5'-ACTCGAGTCGACAGCATCATCTGCAGCC-3'
CK1 δ 3	5'-CGAATTCATGGACTTTGGGCTGGCCAAG-3'
CK1 δ 4	5'-ACTCGAGTCGACGTAGGTGGTACGTCTGTG-3'
CK1 δ 5	5'-CGAATTCATGGAGCTGAGGGTCGGGAAT-3'
CK1 δ 6	5'-ACTCGAGTCGACTCAACGGTGCAGCCGCA-3'
CK1 δ 7	5'-ACTCGAGTCGACTGAGGTAGGGTAAGG-3'
CK1 δ 8	5'-CGAATTCATGGAGCGGGAACGCCG-3'
CK1 δ 9	5'-CCATGGCTCCGGCCGTCTGCGGGGAACC-3'
CK1 δ 10	5'-ACTCGAGTCGACTCAAGGAGAGGTGTTGGC-3'
CK1 δ 11	5'-CCATGGCCCGAATCCAGCCACTCGTGGC-3'
CK1 δ 12	5'-ACTCGAGTCGACTCAATGTGAGGTAGGGGT-3'
CK1 δ 13	5'-ACTCGAGTCGACTCATGGGGGAGCCACTTC-3'
CK1 δ 14	5'-CCATGGCTCCCCAACGCCCTTACCCT-3'
CK1 δ 15	5'-CCATGGCCCAACACCTCTCCTAGACCCTG-3'
CK1 δ 16	5'-ACTCGAGTCGACTCAACGGTGCAGCCGATGCCAC-3'
CK1 δ 17	5'-CCATGGCCCGAATCCAGCCGCTCGTGGC-3'
CK1 δ 18	5'-CCATGGCTCGTGGCTCCCTTCTGCAGCT-3'
CK1 δ 19	5'-CCATGGCCCGCCGCTCTGCGGGGAACCCAG-3'
CK1 δ 20	5'-CGAGAACGAAAGTGGCTATGCGGCTGCACCCTGGG-3'
CK1 δ 21	5'-GCTCTTGCCTTCCACCGATACGCCGAGCTGGCACCC-3'
CK1 δ 22	5'-GATATCATGTACCATACGATGTTCCAGATTACGCTTTCATATGGCGATGGAGCTGAGG-3'
CK1 δ 23	5'-AGATCTTCAGTAGGTGGTACG-3'
CK1 δ 24	5'-GGTACCATGGAGGAGCAGAAGCTG-3'
CK1 δ 25	5'-GTCGACTCAGTAGGTGGTACGCTGTGG-3'

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

FP, fusion protein

Primer pair	CK1 δ amplified (bp)	pGEX vector name	GST-CK1 δ 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	GST-CK1 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	GST-CK1 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-CK1 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 1-428,S370A	GST-CK1 δ 1-428,S370A (FP1023)

protein kinase) as the main cellular kinase that phosphorylates CK1 δ , predominantly at Ser³⁷⁰. In addition, we have demonstrated that Akt (protein kinase B), CLK2 (CDC-like kinase 2) and PKC (protein kinase C) α also phosphorylate CK1 δ at Ser³⁷⁰, as well as at other sites within amino acids 305-375 *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-p53 1-64 *in vitro* and increased the formation of an ectopic dorsal axis during *Xenopus* development. Inhibition of the interaction of PKA with AKAPs (A-kinase-anchoring proteins) by the synthetic St-Ht31 inhibitory peptide led to a significant up-regulation of the activity of CK1 δ in MiaPaCa-2 cells. Our results 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 wild-type rat CK1 δ (GenBank[®] accession number NM_139060) as a template, and the primers used are listed in Table 1.

The PCR fragments were first cloned into the multiple cloning site of pCDNA-3.1/V5-His-TOPO (Invitrogen) and were 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 \rightarrow GC) of rat CK1 δ , was constructed using the QuikChange site-directed mutagenesis kit according to manufacturer's instructions (Stratagene). The plasmid pGEX2T-CK1 δ 1-428 served as a template and the

complementary primers used were CK1 δ 20 (5' primer) and CK1 δ 21 (3' primer) (see Tables 1 and 2).

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

Rat mutant CK1 δ S370A was amplified by PCR using pGEX2T-CK1 δ 1–428,S370A (fusion protein 1021) as a template and the primers CK1 δ 22 (5' primer) and CK1 δ 23 (3' primer) (see Tables 1 and 2), and was cloned into the pcDNA-3.1/V5-His-TOPO vector, generating pcDNA-3.1-HA-mutant CK1 δ S370A (fusion protein 1102).

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

All plasmids were sequenced at GATC Biotech.

Cell lines

Cells (MiaPaCa-2 [35], BxPc3 [36], Colo357 [37] and PancTu1 [38]) were grown in an 1:1 (v/v) mixture of DMEM (Dulbecco's modified Eagle's medium) and RPMI 1640 medium supplemented with 10% (v/v) FCS (fetal calf serum), 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified 5% CO₂ atmosphere at 37°C.

Antibodies

The rabbit polyclonal antibody against PKA (1 mg/ml; Upstate) and the mouse monoclonal CK1 δ -specific antibody, 128A (4 mg/ml; ICOS Corporation), were used. HRP (horseradish peroxidase)-conjugated goat anti-(rabbit IgG) and goat anti-(mouse IgG) antibodies were purchased from GE Healthcare.

Cell treatment, labelling and cell lysis

MiaPaCa-2 cells were treated with 5 μ M H89 (Calbiochem), a PKA-specific inhibitor, 20 μ M forskolin (Calbiochem), a PKA activator, 10 μ M St-Ht31 inhibitory peptide (Promega) or 10 μ M St-Ht31P control peptide (Promega). Sub-confluent MiaPaCa-2 cells were labelled with 2 mCi of [³²P]P_i/ml (GE Healthcare) in phosphate-free DMEM supplemented with 5% (v/v) phosphate-free FCS in the presence of either H89 (5 μ M) or forskolin (20 μ M) for 2 h. Cells were then washed in ice-cold PBS and lysed in sucrose lysis buffer [20 mM Tris/acetate, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM benzamidine, 4 μ g/ml leupeptin, 30 μ g/ml aprotinin and 0.1% (v/v) 2-mercaptoethanol] or NP40 (Nonidet P40) lysis buffer [50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 1% NP40, 5 mM DTT (dithiothreitol), 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 for 30 min at 4°C. The cell lysate was centrifuged at 15 000 g for 20 min at 4°C, the supernatant was passed through a 0.40 μ m-pore-size filter and 3 mg of total protein was applied to an anion-exchange column (Resource-Q) attached to an ETTAN purifier (GE Healthcare). 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% (v/v) glycerol,

0.04% Brij-35, 1 mM benzamidine, 4 μ g/ml leupeptin and 0.1% (v/v) 2-mercaptoethanol.

Western blot analysis

Cells were lysed in NP40 lysis buffer, extracts were clarified by centrifugation at 15 000 g for 20 min at 4°C and the protein concentration of the lysates was determined using the BCA (bicinchoninic acid) protein assay (Pierce). From this determination, 75 μ g of the protein extract was resolved on SDS/PAGE (12.5% gels) and transferred on to a Hybond C super nitrocellulose blotting membrane (GE Healthcare). The membranes were blocked in TBS containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk for 1 h before being probed with either the polyclonal rabbit antibody against PKA (1:1000 dilution) or with the monoclonal mouse antibody 128A (1:5000 dilution) against CK1 δ . Immunocomplexes were detected by incubation for 45 min with HRP-conjugated goat anti-(rabbit IgG) or anti-(mouse IgG) (1:1000 dilution), followed by ECL[®] (enhanced chemiluminescence) detection (GE Healthcare).

Production and purification of GST fusion proteins

The production and purification of the GST fusion proteins (fusion proteins 267, 380, 449, 882, 894, 897, 898, 1001, 1003, 1004, 1006, 1011, 1012, 1013, 1014, 1020, 1021, 1022 and 1023) were carried out as described previously [23].

In vitro kinase assays and phosphopeptide analysis

In vitro kinase assays were carried out in the absence or presence of 5 μ M H89 (PKA inhibitor), 200 nM deguelin (Akt inhibitor), or 1 μ M calphostin C (PKC α inhibitor) as described previously [34]. Different GST-CK1 δ fusion proteins, GST-p53 1–64 (fusion protein 267) and GST-p53 1–85,S4A/S6A/S9A (fusion protein 380), the CK1 peptide substrate (Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg) (Promega) [39] and β -casein (Sigma), were used as substrates. Recombinant PKA (Calbiochem), purified catalytic subunit of PKA from porcine heart (Sigma), recombinant human PKC α (amino acids 1–672) (Biomol), recombinant human Akt (amino acids 106–480) (Biomol), recombinant human CLK2 (Invitrogen) or single fractions of fractionated MiaPaCa-2 cell extracts were used as sources of enzyme. Phosphorylated proteins were resolved by SDS/PAGE and the protein bands were visualized by autoradiography. Where indicated, the phosphorylated protein bands were excised and quantified by Cherenkov counting.

Phosphopeptide analyses of *in vitro* and *in vivo* labelled proteins were performed as described earlier [23].

RNA synthesis

For microinjection experiments, run-off transcripts of rat wild-type 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 synthesized using a T7 mMACHINE[®] kit (Ambion).

Xenopus microinjection

Xenopus embryos were microinjected into both ventral blastomeres at the four-cell stage with the indicated amount of RNA. Secondary axis formation was analysed at stages 22 and 26 of development. Staging was determined as described by Nieuwkoop and Faber [40].

RESULTS

CK1 δ activity differs in pancreatic tumour cell lines

It has been suggested previously [33] that the differences in the activity of CK1 δ in extracts of various organs from young adult Balb/c mice might be influenced by site-specific phosphorylation of CK1 δ . To investigate this further, the activity of CK1 δ was analysed in fractionated extracts from apoptosis-resistant pancreatic tumour cell lines, all expressing similar protein levels of CK1 δ . The kinase activity in fractionated BxPc3, MiaPaCa-2, PancTu1 and Colo357 cell extracts, which eluted at 220–240 mM NaCl and was inhibited by the CK1-specific inhibitor IC261 [41], differed in concentration by up to 6-fold (see Supplementary Figure 1S at <http://www.BiochemJ.org/bj/406/bj4060389add.htm>). These results suggested that post-translational modifications of CK1 δ were involved in modulating the activity of CK1 δ , and this was particularly true for site-specific phosphorylation.

Cellular kinases phosphorylate CK1 δ at Ser³⁷⁰

The C-terminal domain of CK1 δ plays an important role in 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 δ 305–375 as a substrate. A major kinase peak was detected in fractions eluted between 160 and 190 mM NaCl (Figure 1A), indicating that GST-CK1 δ 305–375 can be phosphorylated by cellular kinases *in vitro*.

In order to identify the CK1 δ CK (CK1 δ C-terminal-targeted protein kinase)-dependent phosphorylation site or sites present within the C-terminus of CK1 δ , additional GST-CK1 δ fusion proteins consisting of amino acids 330–375, 305–357 and 353–375 were constructed (Tables 1 and 2) and used as substrates for phosphorylation by CK1 δ CK. Although cellular CK1 δ CK (phosphorylation activity for CK1 δ CK peaked in fraction 10) phosphorylated the truncated GST-CK1 δ fusion proteins GST-CK1 δ 330–375 and GST-CK1 δ 353–375 to a similar extent (96 and 98% respectively) compared with GST-CK1 δ 305–375 (100%), CK1 δ CK phosphorylated GST-CK1 δ 305–357 approx. 38% compared with GST-CK1 δ 305–375 (Figure 1B). Taken together, these results suggest that the major phosphorylation site or sites targeted by cellular kinases are localized within amino acids 353–375 of CK1 δ .

A Scansite search (<http://scansite.mit.edu>) revealed that Ser³⁷⁰ might be the major phosphorylation site within amino acids 353–375 of CK1 δ , which is targeted by cellular kinases, including Akt, PKA, PKC α / β / γ / ζ and CLK2. Therefore GST-CK1 δ 353–375,S370A, containing a serine to alanine mutation at position 370 was generated and used as a substrate for *in vitro* kinase assays. As shown in Figure 1(B), the degree of phosphorylation of GST-CK1 δ 353–375,S370A was approx. 4-fold lower compared with that of GST-CK1 δ 353–375. These results 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 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) were used in the *in vitro* kinase assays. The results shown in Figure 2(A) indicated that Akt predominantly phosphorylated Ser³⁷⁰ *in vitro*.

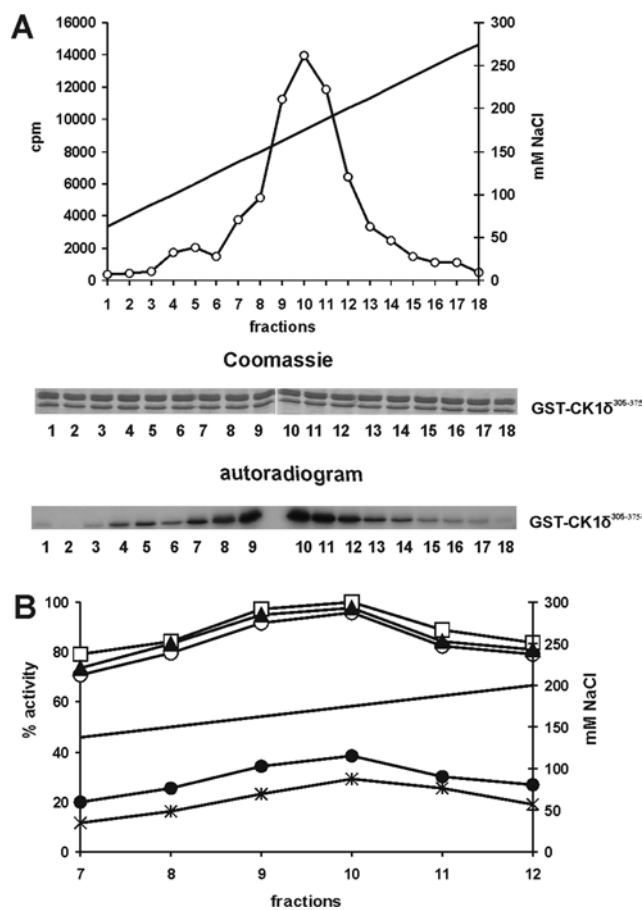


Figure 1 Characterization of CK1 δ CK activities in MiaPaCa-2 cells

(A) Fractionation of CK1 δ CK activity 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 Resource-Q column using a linear ascending gradient of NaCl. Kinase assays were performed using equal amounts of GST-CK1 δ 305–375 as a substrate and single fractions of fractionated MiaPaCa-2 cell extracts as the source of enzyme. Proteins were separated by SDS/PAGE and the phosphorylated proteins were detected by autoradiography. Quantification of phosphate incorporation into GST-p53 1–64 was measured by Cerenkov counting. A major kinase activity, which phosphorylated GST-CK1 δ 305–375 (FP1006), eluted between 160 and 190 mM NaCl (fractions 9–11). (B) Localization of phosphorylation sites targeted by CK1 δ CK activity. *In vitro* kinase assays were performed using fractions 7–12 of fractionated MiaPaCa-2 cell extracts as the source of enzyme activity and fusion proteins GST-CK1 δ 305–375 (FP1006), GST-CK1 δ 330–375 (FP1011), GST-CK1 δ 305–357 (FP1012), GST-CK1 δ 353–375 (FP1022) and GST-CK1 δ 305–375,S370A (FP1021) as substrates. The highest cellular kinase activity (100%) was detected in fraction 10 when using GST-CK1 δ 305–375 (FP1006) as substrate. □, GST-CK1 δ 305–375. ○, CK1 δ 330–375. ▲, CK1 δ 353–375. *, CK1 δ 353–375,S370A. ●, CK1 δ 305–357. Solid line, NaCl concentration. FP, fusion protein.

In contrast, Ser³⁷⁰ was only weakly phosphorylated by PKC α and Clk2 *in vitro*. The major phosphorylation sites targeted by these two kinases were located within amino acids 305–353 of CK1 δ (Figure 2A).

Incubation of a series of GST-CK1 δ fusion proteins (GST-CK1 δ 305–375, GST-CK1 δ 330–375, GST-CK1 δ 305–357, GST-CK1 δ 318–351,S318A, GST-CK1 δ 305–343, GST-CK1 δ 341–375, GST-CK1 δ 353–375,S370A and GST-CK1 δ 353–375) with the catalytic subunit of PKA purified from porcine heart and [γ -³²P]ATP confirmed that Ser³⁷⁰ was 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 δ 305–375 as substrate. The result of three independent experiments indicated that approx. 1.5 mol of

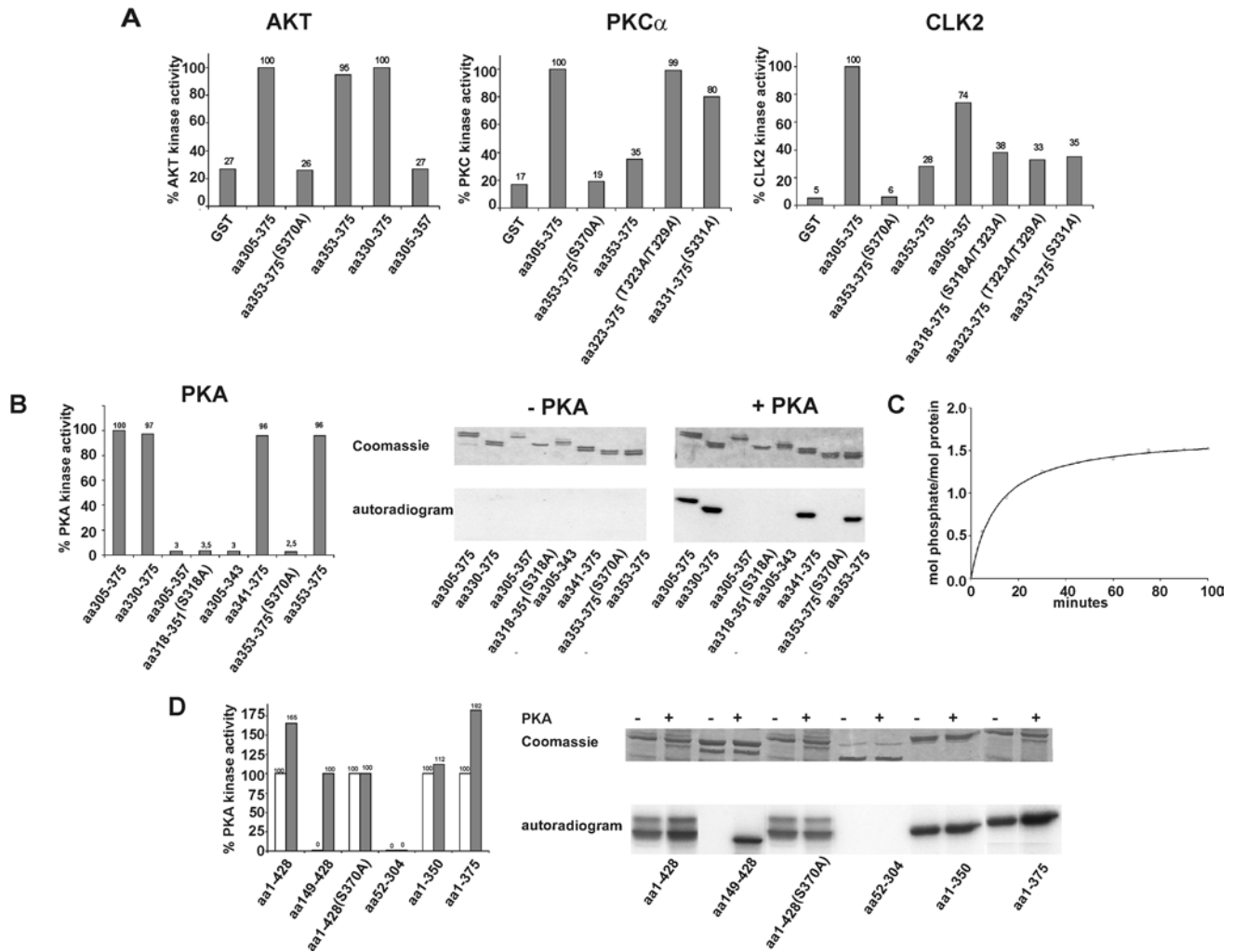


Figure 2 Phosphorylation of CK1 δ by cellular kinases *in vitro*

(A) Phosphorylation of CK1 δ by Akt, PKC α and CLK2. *In vitro* kinase assays were performed using recombinant human PKC α (amino acids 1–672), recombinant human Akt (amino acids 106–480) and recombinant human CLK2 as sources of enzyme, 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) Characterization of the site-specific phosphorylation of CK1 δ by PKA. *In vitro* kinase assays were performed using the catalytic subunit of PKA purified from porcine heart as the source of enzyme and GST–CK1 δ 305–375 (FP1006), 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 resolved by SDS/PAGE (12.5% gels) and stained with Coomassie Brilliant Blue. 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* kinase assays were performed using the catalytic subunit of PKA purified from porcine heart as the source of enzyme and GST–CK1 δ 1–428 (FP449), GST–CK1 δ 149–428 (FP894), GST–CK1 δ 1–428,S370A (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 Blue-stained gel and the quantification analysis presented are of a representative experiment. Open bars, without PKA; closed bars, with PKA. aa, amino acid; FP, fusion protein.

phosphate were incorporated per mol of protein, suggesting that there is one major phosphorylation site within amino acids 305–375 of CK1 δ that is phosphorylated by PKA *in vitro* (Figure 2C).

To examine whether additional serine and 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 δ 149–428 were used as substrates. All GST–CK1 δ fusion proteins containing Ser³⁷⁰ (GST–CK1 δ 1–428, GST–CK1 δ 149–428 and 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, the degree of phosphorylation increased up to 82% in the presence of purified PKA. In contrast with this, 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 δ 1–428,S370A fusion protein was used as a substrate, no change in the phosphorylation status of CK1 δ was 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, CK1 δ CK activity was assayed in the absence and presence of kinase inhibitors specific for PKA, PKC α or Akt, using GST–CK1 δ 305–375 as the substrate. As demonstrated in Figure 3(A), a 65% reduction in CK1 δ CK activity was observed in the presence of the PKA-specific inhibitor H89 (5 μ M),

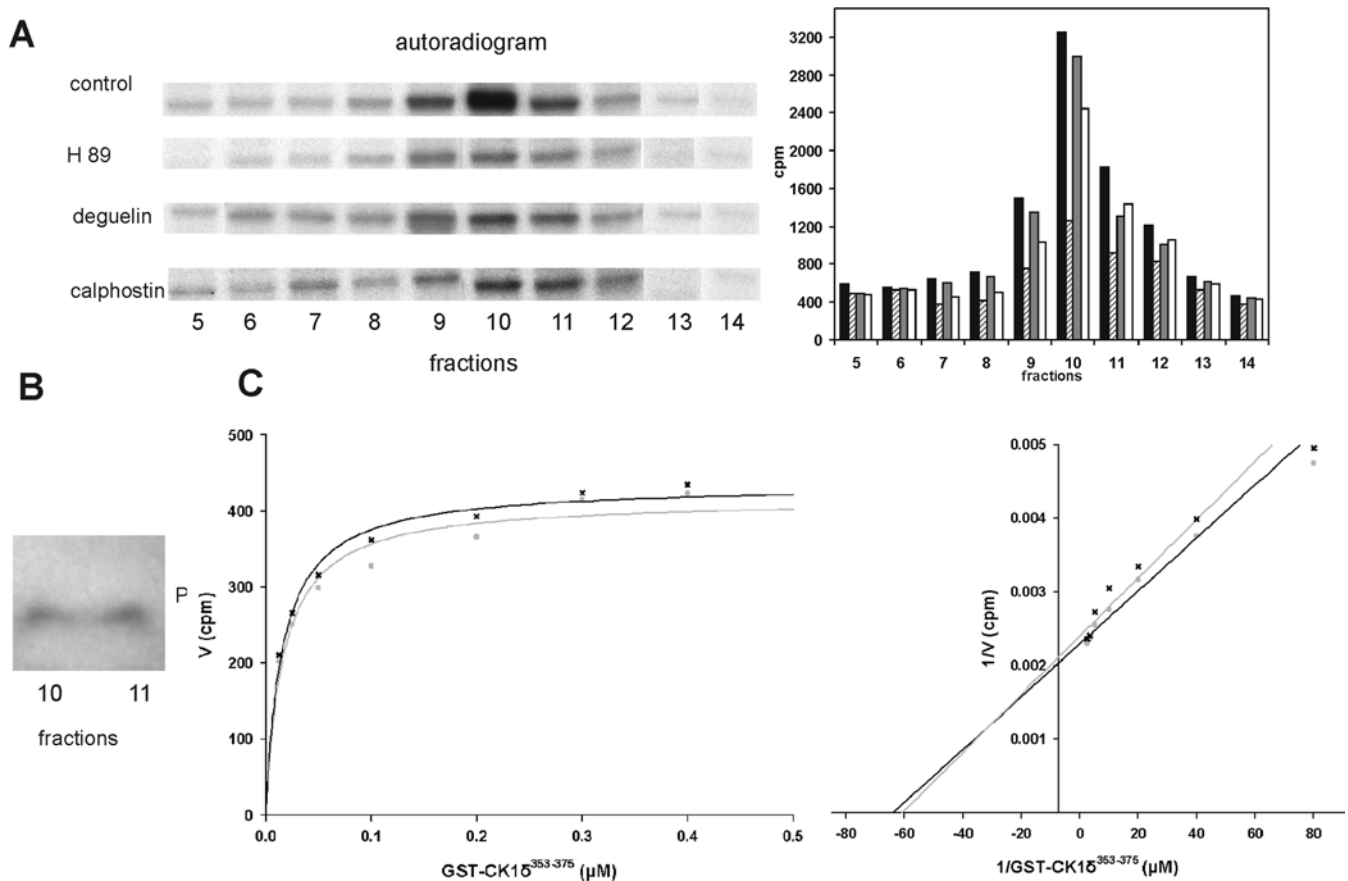


Figure 3 PKA is the major CK1 δ CK activity present in the kinase peak fraction of fractionated MiaPaCa-2 cells

(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 μM), the Akt-specific inhibitor degeulin (200 nM) or the PKC-specific inhibitor calphostin C (1 μM), using GST-CK1 δ 305–375 (FP1006) as the 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 by 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 δ 353–375 (FP1022) as a substrate. The results indicate that the K_m and V_{max} values of CK1 δ CK (grey dots) and PKA (black crosses) are nearly identical. FP, fusion protein.

whereas, in the presence of the PKC-specific inhibitor calphostin C, (1 μM) or the Akt-specific inhibitor degeulin (200 nM), the reduction in CK1 δ CK activity was much lower (25 and 9% respectively).

As PKA seems to be the major CK1 δ CK-active kinase present in the kinase peak fractions (Figure 1A) and was clearly detected in the kinase peak (fractions 10 and 11) by Western blot analysis (Figure 3B), the kinetic parameters of CK1 δ CK activity were compared with those of recombinant PKA, using the GST-CK1 δ 353–375 fusion protein as the 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 in the catalytic activity of CK1 δ *in vitro*, the kinetic constants of phosphorylation of β -casein and GST-p53 1–64 by GST-CK1 δ 1–428 and GST-CK1 δ 1–428,S370A were calculated experimentally. GST-CK1 δ 1–428,S370A had lower K_m and higher V_{max} values

compared with those of GST-CK1 δ 1–428 (see Supplementary Figure 2S at <http://www.BiochemJ.org/bj/406/bj4060389add.htm>). These observed differences revealed that CK1 δ 1–428,S370A had a higher affinity for phosphorylating β -casein and GST-p53 1–64 compared with that of GST-CK1 δ 1–428, which was also indicated by the efficiency parameter (V_{max}/K_m ratio) in Supplementary Figure 2S(C).

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

So far, our results clearly indicate that Ser³⁷⁰, 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 (fusion protein 449) was used as a source of enzyme activity. CK1 δ was pre-incubated with [γ -³²P]ATP for 15 min before the addition of GST-p53 1–64 (fusion protein 267) as a substrate, either alone or in combination with PKA for the indicated time points (see Supplementary Figure 3S, at <http://www.BiochemJ.org/bj/406/bj4060389add.htm>).

As shown in Supplementary Figure 3S, CK1 δ -mediated phosphorylation of GST-p53 1–64 (fusion protein 267) was reduced

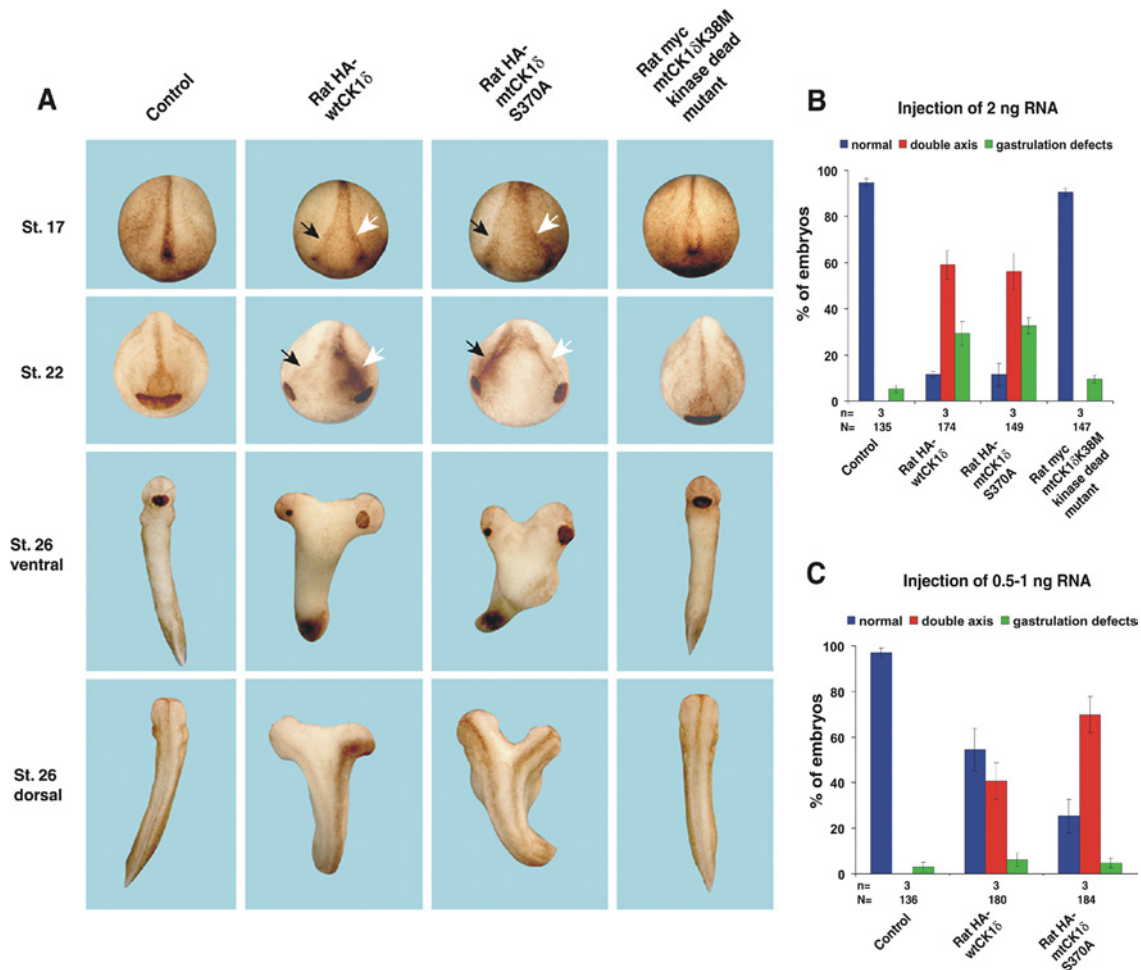


Figure 4 Secondary axis induction in *X. laevis* embryos

X. laevis embryos were injected with wild-type or mutant CK1 δ RNA at the four-cell stage as indicated. Evaluation of secondary axis formation was performed at stage (St.) 22 or 26. **(A)** Different views of embryos are shown. **(B)** Quantification of experiments showed that injection of higher amounts resulted in secondary axis formation and gastrulation movement defects in the case of wild-type CK1 δ and CK1 δ S370A. The kinase-dead mutant, CK1 δ K38M, is inactive in this assay ($n = 3$). N, total number of embryos used in all three experiments. **(C)** Injection of lower amounts of RNA and selection of embryo batches with low gastrulation movement phenotype indicated that CK1 δ S370A has a higher capacity to induce secondary axis formation compared with wild-type CK1 δ ($n = 3$). N, total number of embryos used in all three experiments.

up to 80% in the presence of PKA compared with that observed in the absence of the catalytic subunit of PKA. Phosphorylation of GST-p53 1–64 by PKA could be excluded as *in vitro* kinase assays demonstrated that PKA did not phosphorylate GST-p53 1–64 (results not shown).

Effects of CK1 δ S370A in *Xenopus* embryogenesis

Different CK1 isoforms have been implicated in the Wnt signalling pathway. Considering the high affinity of CK1 δ S370A for phosphorylating both β -casein and GST-p53 1–64 *in vitro*, we analysed the effects of this mutant on Wnt/ β -catenin signalling *in vivo* using the *Xenopus* secondary axis induction assay.

In vitro-transcribed RNA encoding HA-tagged rat wild-type CK1 δ , mutant CK1 δ S370A or myc-tagged mutant CK1 δ K38M were microinjected into both ventral cells of four-cell-stage *Xenopus* embryos. The kinase-dead mutant CK1 δ K38M did not perturb embryonic development, whereas high doses (2 ng) of either wild-type CK1 δ or CK1 δ S370A resulted in the formation of an ectopic dorsal axis (Figures 4A and 4B), as well as gastrulation movement defects. To identify any difference in

the biological activity of both constructs, we lowered the concentration of injected RNA in subsequent experiments and selected for embryos which displayed a low gastrulation phenotype (not more than 10%, as this can occur non-specifically in egg batches of low quality or as an eventual result of injection of higher quantities of RNA). At lower amounts of microinjected mRNA, the secondary axis phenotype was demonstrated more in embryos expressing exogenous HA-tagged CK1 δ S370A than in embryos expressing wild-type CK1 δ (Figure 4C).

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 modulation of the activity of PKA should influence the phosphorylation status of CK1 δ . For this purpose, MiaPaCa-2 cells were treated either with 5 μ M H89 (PKA-specific inhibitor) or with 20 μ M forskolin (PKA activator) in the presence of 2 mCi [³²P]P_i for 2 h. Cells were lysed and phosphorylated CK1 δ was immunoprecipitated, isolated, digested with trypsin and oxidized. The resulting tryptic phosphopeptides were separated by

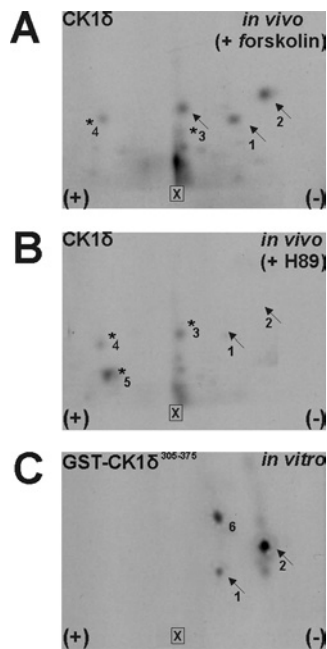


Figure 5 Phosphopeptide analyses of CK1 δ and GST-CK1 δ 305–375

GST-CK1 δ 305–375 and [32 P]-labelled CK1 δ isolated from MiaPaCa-2 cells were treated with either 20 μ M forskolin (A) or 5 μ M H89 (B) for 2 h and were oxidized and digested with trypsin (see Materials and methods section). 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 that are identical in the *in vitro* and the *in vivo* maps. (A) CK1 δ -specific phosphopeptides from forskolin-treated MiaPaCa-2 cells. (B) Phosphopeptides of CK1 δ from H89-treated MiaPaCa-2 cells. (C) Phosphopeptides from the GST-CK1 δ 305–375 fusion protein phosphorylated by PKA *in vitro*. X, loading point.

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 that had been treated with H89 (Figure 5B), suggesting that PKA is the cellular kinase responsible for the phosphorylation of amino acid residues within these two peptides. Other migrated peptides (labelled peptides 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.

Subcellular localization of PKA is important for modulating the activity of CK1 δ

Compartmentalization of signal transduction molecules, such as kinases and phosphatases, occurs by anchoring of proteins to scaffold proteins. This allows substrate phosphorylation and dephosphorylation in conjunction with various kinases and phosphatases, which results in compartment-specific regulation of the substrate.

It has been reported that the 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 AKAPs can be blocked by the membrane-permeant AKAP-inhibitory peptide St-Ht31 [42,44]. Therefore we investigated whether disruption of the PKA–AKAP complex by the anchoring-disrupting peptide St-Ht31 [45,46] could influence the activity

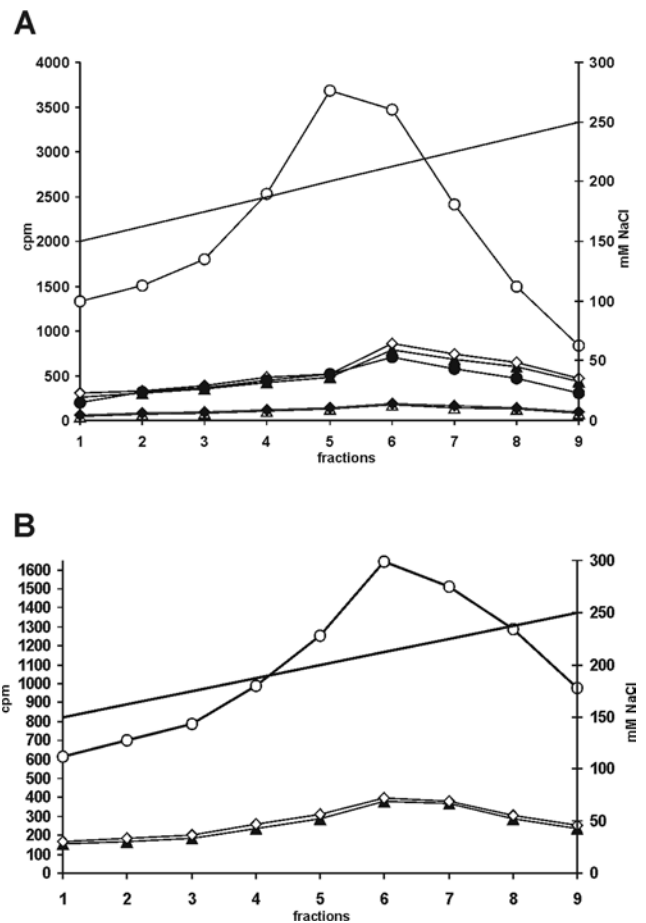


Figure 6 Inhibition of the interaction of PKA with AKAPs is accompanied by an increase in CK1 δ activity

Equal amounts of protein (3 mg) from MiaPaCa-2 cell lysates which had been untreated, treated with 10 μ M St-Ht31 control peptide or with 10 μ M St-Ht31-specific inhibitory peptide were fractionated (see Materials and methods section). The proteins were eluted using a linear gradient of increasing NaCl concentrations (represented as a solid line). (A) The CK1 δ -specific kinase activity was determined using GST-p53 1–64 (FP267) and GST-p53 1–84,S4A/S6A/S9A fusion proteins (FP380) as substrates. When GST-p53 1–64 was used as a substrate: \circ , cells treated with St-Ht31 inhibitory peptide; \diamond , cells treated with St-Ht31 control; \blacktriangle , untreated cells. When GST-p53 1–84,S4A/S6A/S9A was used as a substrate: \bullet , cells treated with St-Ht31 inhibitory peptide; \blacklozenge , cells treated with St-Ht31 control; \triangle , untreated cells. (B) CK1 δ -specific kinase activity was also determined using the CK1 peptide substrate Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg. \circ , Cells treated with St-Ht31 inhibitory peptide; \diamond , cells treated with St-Ht31 control peptide; \blacktriangle , untreated cells. FP, fusion protein.

levels of CK1 δ . For this purpose, MiaPaCa-2 cells were treated with 10 μ M of either St-Ht31 or 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-p53 1–64 (fusion protein 267) (Figure 6A) were used as substrates, the CK1 δ activity increased 3–6-fold in cells treated with the St-Ht31 inhibitory peptide compared with untreated cells and cells treated with the St-Ht31P control peptide (Figure 6).

Differences in the CK1 δ kinase activity were observed in cells treated with the St-Ht31 inhibitory peptide when GST-p53 1–85,S4A/S6A/S9A (fusion protein 380) was used as a substrate, compared with untreated cells and cells treated with the St-Ht31P control peptide (Figure 6A). However, as expected, the CK1 δ -mediated phosphorylation of GST-p53 1–85,S4A/S6A/S9A was

significantly lower compared with that of GST-p53 1–64, owing to the mutation of the majority of the CK1 δ phosphorylation sites (S4A/S6A/S9A) 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 at different levels [15,24,26,28,48,49].

Using a biochemical approach to clarify the role of cellular kinases in modulating CK1 δ CK activity, MiaPaCa-2 cell lysates were fractionated and the kinase activity was detected in fractions eluted in 160–190 mM NaCl. The use of various C-terminal-truncated wild-type and mutant GST-CK1 δ fusion proteins as substrates revealed that Ser³⁷⁰ is the main residue targeted by CK1 δ CK for phosphorylation. Ser³⁷⁰ is a potential phospho-acceptor site for phosphorylation by several putative kinases, including 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, whereas the key targeted sites of PKC α and CLK2 lie within amino acids 305–353 of CK1 δ . However, additional experiments are necessary to identify these 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 results strongly suggest that this serine residue is predominantly phosphorylated by PKA. PKA co-eluted with the peak of the CK1 δ CK activity, as verified by Western blot analysis, suggesting that PKA provides CK activity. 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 in kinase activity. Comparison of the kinetic parameters of recombinant PKA with those of CK1 δ CK present in the kinase peak fraction indicated nearly identical parameters. 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, suggesting that PKA is responsible for the phosphorylation of Ser³⁷⁰. 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 suggested that CK1 δ is phosphorylated *in vivo* at the same sites which are phosphorylated by PKA *in vitro*.

In addition, phosphorylation of CK1 δ by PKA reduced the ability of CK1 δ to phosphorylate GST-p53 1–64 up to 80 %. This result indicated that site-specific phosphorylation may be involved in the modulation of CK1 δ activity.

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 1–428,S370A. Our results show that the GST-CK1 δ 1–428,S370A mutant exhibits lower K_m and higher V_{max} values when compared with those of GST-CK1 δ 1–428, when β -casein and GST-p53 1–64 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 the 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 with the expression of wild-type CK1 δ , whereas the kinase-dead mutant (CK1 δ K38M) failed to induce axis formation.

In addition, our results strongly support the idea that CK1 δ and PKA interact physiologically. This is due to the observation that inhibition of the interaction of PKA with AKAPs by the St-Ht31 inhibitory peptide [45,51] resulted in an increase in 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 reduced phosphorylation of Ser³⁷⁰. Furthermore, cellular phosphatases associated with AKAPs [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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