Regulation of Casein Kinase I ε and Casein Kinase I δ by an in Vivo Futile Phosphorylation Cycle*

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Casein kinase I δ (CKIδ) and casein kinase I ε (CKIε) have been implicated in the response to DNA damage, but the understanding of how these kinases are regulated remains incomplete. In vitro, these kinases rapidly autophosphorylate, predominantly on their carboxyl-terminal extensions, and this autophosphorylation markedly inhibits kinase activity (Cegielska, A., Gietzen, K. F., Rivers, A., and Virshup, D. M. (1998) J. Biol. Chem. 273, 1357–1364). However, we now report that while these kinases are able to autophosphorylate in vivo, they are actively maintained in the dephosphorylated, active state by cellular protein phosphatases. Treatment of cells with the cell-permeable serine/threonine phosphatase inhibitors okadaic acid or calyculin A leads to rapid increases in kinase intramolecular autophosphorylation. Since CKI autophosphorylation decreases kinase activity, this dynamic autophosphorylation/dephosphorylation cycle provides a mechanism for kinase regulation in vivo.

The casein kinase I (CKI) gene family contains two major subgroups that regulate cytoplasmic and nuclear processes, respectively. The nuclear family appears involved in the response to DNA damage in mammals and yeast, whereas the cytoplasmic members are involved in membrane structure and bud morphogenesis (1–5). The founding member of the nuclear family, HRR25, was first cloned in a screen for budding yeast bud morphogenesis (1–5). The founding member of the nuclear family appears involved in the regulation of DNA repair (6, 7). In Saccharomyces cerevisiae with a deletion of the HRR25 gene (7). A number of the substrates identified to date for the CKI family also support a role for the kinase in regulation of DNA repair and replication. For example, phosphorylation of SV40 large T antigen blocks its ability to unwind the SV40 origin of replication (8–11). CKI also phosphorylates several isoforms of the rad24 and rad25-related 14–3-3 proteins, regulating their ability to bind to substrate proteins (12). Purified CKIε can phosphorylate the carboxyl terminus of IκBα, potentially regulating its degradation rate (10, 13). Hrr25 protein binds to and phosphorylates the yeast transcription factor Swi6 (14). Most recently, CKIε and CKIδ have been identified as the cellular kinases that constitutively phosphorylate the extreme amino terminus of p53 (5).

CKI genes appear widely and constitutively expressed, but despite the progress in defining a role for CKI isoforms in the cell, a detailed understanding of the regulation of CKI activity is lacking. Several lines of evidence suggest the carboxyl-terminal tails of CKIδ and CKIε contain an important autoregulatory domain. First, CKIε, with a 124-amino acid carboxyl-terminal extension, complements hrr25Δ yeast, while CKIδ, with a 24-amino acid extension, does not (7). CKIδ and CKIε both display a significant increase in specific activity when the carboxyl terminus is removed (10, 15). More strikingly, CKIε rapidly autophosphorylates in vitro in an intramolecular reaction that substantially inhibits kinase activity toward a number of protein substrates. CKIε, but not a mutant lacking the carboxyl terminus, can then be activated up to 20-fold by dephosphorylation (10). These studies suggest that changes in the phosphorylation state of CKIδ and CKIε carboxyl termini in vivo may be an important mechanism for regulation of the kinase activity.

In the present study, we investigated the autoregulatory autophosphorylation of CKIε and CKIδ in vivo. Unexpectedly, we found that in cultured cells and in tissues, these kinases are actively maintained in the dephosphorylated state. While the kinases are indeed able to rapidly autophosphorylate in vivo as well as in vitro, they are maintained in the dephosphorylated state in vivo by rapid constitutive dephosphorylation by cellular protein phosphatases sensitive to okadaic acid and calyculin A. These results indicate that CKIε and CKIδ utilize ATP in a futile cycle of autophosphorylation and dephosphorylation. Stimuli that regulate the relevant phosphatases may regulate the activity of these kinases in the cell.

EXPERIMENTAL PROCEDURES

Recombinant histidine tagged and untagged CKIε were produced in Escherichia coli as described (7, 10). The monoclonal antibody directed against the CKIε carboxyl-terminal domain, CKIε mAb, was purchased from Transduction Laboratories, Lexington, KY. This antibody preferentially recognizes CKIε although it cross-reacts to a lesser extent with CKIδ. The specific anti-CKIδ monoclonal antibody 128A was the generous gift of M. Hoekstra, Icos Corp., Bothell, WA. The polyclonal antiserum UT31, raised against the amino terminus of CKIε, has been previously described (10). Immunoblots were visualized with the enhanced chemiluminescence (ECL) kit from Amersham Pharmacia Biotech.

Cell Treatments with Phosphatase Inhibitors—NIH 3T3 mouse fibroblasts were plated in 35-mm round dishes and grown in Dulbecco’s
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modified Eagle’s medium (DMEM) with 10% fetal calf serum at 37 °C in a 5% CO₂ incubator. At 70% confluence, cells were treated with calyculin A or okadaic acid in Me₂SO at indicated times and concentrations. Me₂SO was added as control in equal amounts to all untreated cells. At the indicated times, the growth medium was removed, and the cells were lysed with 1× SDS-polyacrylamide gel electrophoresis (PAGE) loading sample buffer (10% glycerol, 50 mM Tris, pH 6.8, 2% SDS, 3% β-mercaptoethanol, and 0.1% bromphenol blue), boiled for 3 min, and separated by SDS-PAGE. Proteins were electrochemically transferred to nitrocellulose and immunoblotted with the indicated antibodies.

Phosphorylation and Dephosphorylation Reactions—HeLa extracts were prepared as described (16). In vitro phosphorylation or dephosphorylation reactions contained 25 μg of HeLa extract with 4 μM adenosine triphosphate, in the presence or absence of phosphatase inhibitors (100 nm calyculin A, 250 nm okadaic acid, and 50 μM β-glycerophosphate), where indicated, in 30 mM Hepes, pH 7.5, and 7 mM magnesium chloride and were incubated at 37 °C for 30 min. Phosphatase reactions contained 200 ng of protein phosphatase 2A catalytic subunit (PP2A).

Rat Tissues—The indicated rat organs were excised, snap-frozen in liquid nitrogen, and then weighed and ground with a chilled mortar and pestle. Tissues were homogenized with a Polytron (Brinkmann) using 3 ml/gm tissue of 50 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, and 3 μg/ml pepstatin, or in a separate experiment, by addition of 100 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 3% β-mercaptoethanol, and 0.1% bromphenol blue, boiled for 3 min, and separated by SDS-PAGE. Proteins were electrochemically transferred to nitrocellulose and immunoblotted with the indicated antibodies.

Mammalian Expression Constructs—The protein coding sequences of CKIα and CKIε were cloned in frame directly downstream of the hemagglutinin (HA) epitope (YPYDVPDYA) coding sequence, contained in pBluescript-HA (the generous gift of F. Brown and E. Leibold, University of Utah). The HA-tagged CKI open reading frames were subsequently excised with HindIII and XbaI and cloned into the same sites of pRcCMV (Invitrogen). The resulting constructs were pV367 (CMV-HA-CKIα) and pV371 (CMV-HA-CKIε). Similar constructs, pV368 (CMV-HA-CKIαK46A) and pV372 (CMV-HA-CKIεK38R), were generated using kinase-deficient point mutations of each isoform (7). For immunoprecipitation studies, pV405 (4HA-CKIα) and pV406 (4HA-CKIεK38R) containing four copies of the HA epitope were constructed in pCEP4 (Invitrogen) (17).

Transfections—COS-1 (SV40-transformed monkey kidney) cells were grown in DMEM plus 10% supplemented calf serum (Life Technologies, Inc. and Hyclone, respectively), 293 cells (human embryonic kidney cells) were grown in DMEM plus 10% fetal bovine serum (Hyclone). Cells at 50–90% confluence were transfected with 2 μg of plasmid DNA mixed with 6 μl of LipofectAMINE reagent (Life Technologies) following the instructions of the manufacturer. Transfection efficiencies were routinely 40–50% for COS-1 and 293 cells.

Subcellular Fractionation—COS-1 cells were lysed in hypotonic lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 0.5 mM sodium orthovanadate, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μM PMSF) 48 h after transfection. Soluble material was clarified by centrifugation at 1,000 × g for 10 min. To the resulting supernatant, a 1/10 volume of 0.3 M Hepes, pH 7.9, 1.4 mM KCl and 30 mM MgCl₂ was added, and the mixture was centrifuged for 15 min at 14,000 × g. The resulting pellet was designated the membrane fraction and the supernatant was the cytoplasmic fraction. The pellet from the 14,000 × g centrifugation was extracted with high salt buffer containing 20 mM Hepes, pH 7.9, 420 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 25% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μM PMSF. The protein was mixed slowly at 4 °C for 30 min and then clarified by centrifugation for 15 min at 14,000 × g. The resulting supernatant is referred to as the nuclear fraction.

Metabolic Labeling—Transiently transfected 293 cells were metabolically labeled for 5 h in 5% dialyzed calf serum (Life Sciences), 2 μCi/ml H₂¹⁴C, (NEN Life Science Products), and phosphate-free DMEM (Life Sciences) starting at 36 h post-transfection. Calyculin A was not added or added to the transiently transfected cultures at a final concentration of 50 nm during the last 30 min of metabolic labeling. Cultures were lysed by lysis in RIPA buffer (50 mM Tris-HCl, pH 8.6, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM PMSF, 5 mM Na₃VO₄, 20 mM NaF, 250 mM okadaic acid, 20 mM β-glycerophosphate) and clarified by centrifugation at 14,000 × g for 30 min. Soluble extracts containing HA-tagged proteins were immunoprecipitated with 12CA5 mAb and protein A-agarose and analyzed by SDS-PAGE on a 9% gel. Results were visualized by PhosphorImager (Molecular Dynamos).

RESULTS

The phosphorylation state of CKIε was initially assessed by following the shift in electrophoretic mobility of the kinase after SDS-PAGE and immunoblot analysis. As shown in Fig. 1A, recombinant autophosphorylated CKIε (lane 2) displays a marked decrease in electrophoretic mobility compared with PP2Aₐ₉-treated CKIε (lane 1). When log-phase 3T3 cells were lysed in SDS and the mobility of endogenous CKIε was examined by immunoblot analysis, we likewise found that the immunoreactive kinase was predominantly in the high mobility, dephosphorylated state (Fig. 1A, lanes 3 and 8).

In a recent study, we demonstrated that in vitro purified CKIε is capable of rapid autophosphorylation, with a marked reduction in electrophoretic mobility within 5 min of the addition of ATP (10). How is CKIε maintained in the dephosphorylated state in vivo? Two potential mechanisms are, first, the kinase may be unable to autophosphorylate in vivo or, second, it may be dephosphorylated more rapidly than it autophosphorylates. To evaluate the second possibility, that there are cellular phosphatases that actively dephosphorylate CKIε, we treated 3T3 cells with the cell-permeable serine/threonine phosphatase inhibitors okadaic acid and calyculin A and determined their effect on the phosphorylation state of the kinase. As shown in Fig. 1A, lanes 3–15, treatment of cells with either okadaic acid or calyculin A leads to a dose-dependent change in CKIε electrophoretic mobility that reflects a change in phosphorylation. This effect was quite rapid; as seen in Fig. 1B, lane 1, at a dose of 100 nM calyculin A for as little as 15 min led to a marked increase in kinase phosphorylation. Note, too, that the effect is not antibody-dependent since the immunoblot in Fig. 1A was probed with anti-CKIε mAb directed against the carboxyl terminus of CKIε, and in Fig. 1B, lane 1, it was probed with UT31, a rabbit polyclonal antibody that recognizes the amino terminus of CKIε and CKIδ (10). While the CKIε mAb also
recognition of CKIδ, the two kinases appear regulated in a coordinate manner since all the immunoreactive kinase behaved similarly both in the absence and presence of phosphatase inhibitors.

To address the question of whether the in vivo dephosphorylation of the kinases was restricted to transformed, log-phase tissue culture cells or was a general phenomenon, the tissue expression pattern of CKI was first investigated (Fig. 2A). Northern blot analysis indicates several forms of CKI, including CKIα, CKIδ, and CKIε mRNA, are widely and similarly expressed in all human tissues examined, with the highest levels in muscle and brain (Fig. 2A). Protein extracts were then prepared from flash-frozen rat tissues and analyzed by SDS-PAGE and immunoblotting. As seen in Fig. 2B, CKIε in all rat tissues examined was predominantly in the high mobility, dephosphorylated form. Interestingly, the ratio of CKI mRNA to protein varies widely between tissue (e.g., compare the ratio of mRNA to immunoreactive protein in liver and heart), suggesting additional post-translational regulation. Trace amounts of lower mobility immunoreactive species were seen in several tissues after longer exposure times (data not shown), suggesting some turnover of more highly phosphorylated kinase. The results suggest CKIε dephosphorylation is a widespread phenomenon, and is not limited to tissue culture or transformed cells.

To determine whether CKI was dephosphorylated by nuclear or cytoplasmic phosphatases, the subcellular localization of CKIε (and as a control, CKIα) was investigated. COS-1 cells were transiently transfected with hemagglutinin epitope-tagged CKIα2 and CKIε expression constructs. 48 h after transfection, cells were lysed and membrane, soluble, and nuclear fractions were prepared by differential centrifugation as described (see “Experimental Procedures”). The pattern of cross-reacting material present in the fractions from untransfected cells (Fig. 2C, lanes 1–3) confirms that the fractions contain distinct proteins. CKIα2 remained predominantly in the soluble fraction (Fig. 2C, lanes 4–6), while high levels of CKIε were seen in membrane, soluble, and nuclear fractions (Fig. 2C, lanes 7–9). A parallel experiment done with kinase-inactive forms of CKIα2 and CKIε showed an identical pattern of subcellular distribution (data not shown), suggesting that kinase activity is not required for CKIε nuclear localization.

To further characterize the role of cellular phosphatases that catalyze the rapid turnover of phosphoryl groups on CKIε, the fate of the kinase in cell extracts was examined. As shown in Fig. 3, recombinant histidine-tagged CKIε fully autophosphorylates when incubated with ATP in the absence of cell extracts (Fig. 3A, lanes 1 and 2). However, when the same amount of kinase is incubated with 25 μg of HeLa extract, a small increase in kinase mobility is seen (compare Fig. 3A, lane 1 with lanes 3 and 4), indicating removal of residual phosphoryl groups. If the protein phosphatases present in the HeLa extract are inhibited, then the added CKIε rapidly autophosphorylates. (Fig. 3A, lane 5). Similarly, when HeLa extracts are incubated without added recombinant CKIε with or without ATP (Fig. 3A, lanes 6–9), no change in endogenous CKIε electrophoretic mobility is seen unless protein phosphatase inhibitors are first added (compare Fig. 3A, lanes 6 and 7 with lanes 8 and 9). Note that even in the absence of added ATP, sufficient ATP is present in the cell extract to permit autophosphorylation of endogenous CKIε once phosphatase inhibitors are added (Fig. 3A, lane 8). The HeLa phosphatases are very efficient at removing

Fig. 2. CKIε is a widely distributed nuclear and cytoplasmic kinase that is predominantly dephosphorylated in various tissues. A, human tissue expression patterns. A Northern blot of human RNA (CLONTECH) was probed sequentially with 32P-labeled β-actin and CKIε, CKIδ, and CKIα cDNAs (7). mRNA sizes are indicated in kilobases. B, immunoblot analysis of endogenous CKIε. 200 ng of recombinant CKIε (rCKIε) was dephosphorylated with 200 ng of PP2A, (lane 1) or autophosphorylated in the presence of 4 mM ATP for 30 min at 37 °C (lane 2). The kinases and 25 μg each of protein extract from HeLa cells (lane 3) and the indicated rat tissues (lanes 4–8) were separated by 10% SDS-PAGE and immunoblotted with anti-CKIε monoclonal antibody. Recombinant CKIε samples were exposed to film for 20 s, and all other samples were exposed to film for 1 min. The closed circle indicates the position of phosphorylated CKIε, and the open circle indicates the position of dephosphorylated CKIε. Sizes are indicated in kilodaltons. C, CKIε is both nuclear and cytoplasmic. COS-1 cells were transiently transfected with the indicated HA-tagged expression constructs or empty vector and separated into membrane (M), cytosol (C), and nuclear (N) fractions as described under “Experimental Procedures.” Equal cell equivalents were separated by 10% SDS-PAGE and immunoblotted with 12CA5 mAb that recognizes the HA epitope. The positions of CKIα2 and CKIε are indicated by arrows; prominent 12CA5-cross-reacting bands are indicated by an *.
phosphates from CKI; Fig. 3B demonstrates that when histidine-
tagged CKI, autophosphorylated in the presence of $[^{32}P]ATP$, is added back to HeLa extract then 50% of the $^{32}P$-labeled phosphate groups are removed in less than 1 min and 90% are removed by 10 min. Note that fully dephosphorylated histidine-tagged CKI has an electrophoretic mobility of approximately 50 kDa and is not visible in the autoradiograph. This decrease in the $^{32}P$-labeled CKI is due to dephosphorylation and not proteolysis, as incubation of CKI in HeLa extract for as long as 30 min has no effect on protein levels (Fig. 3A). This data is consistent with the hypothesis that casein kinase I α autophosphorylation is ongoing both in vivo and in extracts but is rapidly reversed in vivo and in cell extracts by okadaic acid and calyculin A-sensitive phosphatases.

In order to confirm that CKI phosphorylation in cells and extracts is indeed due to an intramolecular reaction, rather than phosphorylation in trans by other forms of CKI or other cellular kinases, recombinant wild-type CKI or a kinase-inactive CKI mutant (K38R) (7) was incubated with HeLa extract, and its phosphorylation state was assessed by immunoblotting (Fig. 4). While wild-type CKI readily autophosphorylated Fig. 4, lanes 1–4), the kinase-deficient mutant K38R showed no change in electrophoretic mobility despite a 30-min incubation in the presence of ATP and phosphatase inhibitors (lanes 5–8). This result strongly suggests that CKI phosphorylation is due to intramolecular autophasorylation. To confirm this result in vivo, a hemagglutinin-tagged kinase-dead CKI was transiently expressed in 3T3 cells. No change in electrophoretic mobility (i.e. no significant phosphorylation) of the kinase-dead CKI occurred even after treatment of the transfected cells with calyculin A (data not shown). These results demonstrate that CKI autophosphorylates in an intramolecular manner, and additionally suggests that no other cellular kinases present in HeLa extracts phosphorylate CKI in a manner that contributes to changes in electrophoretic mobility.

Two closely related forms of CKI have been identified in mammals: CKIα and CKIβ. These kinases differ by only eight residues in the kinase domain and are about 53% identical over the 124-amino acid carboxyl-terminal regulatory domain. Since the monoclonal antibody directed against the CKI tail cross-reacts with CKIβ, it was important to examine whether CKIβ was specifically maintained in a high mobility, dephosphorylated state in vivo. 3T3 cells were therefore treated with calyculin A or okadaic acid, and cellular extracts were probed with monoclonal antibodies against CKIα and CKIβ. As shown in Fig. 5, CKIβ showed a similar marked decrease in electrophoretic mobility upon phosphatase inhibitor treatment. Interestingly, CKIβ displayed a more rapidly and uniform change in electrophoretic mobility, while CKI appeared to decrease in intensity. While this decrease in CKI immunoreactivity could be due to phosphorylation-regulated proteolysis, we found no change in signal intensity when cells were pretreated with proteosome or other protease inhibitors (data not shown). The fact that the two antibodies gave similar but not identical results supports the conclusion that the CKIα mAb is relatively specific for CKIα.

**DISCUSSION**

Phosphorylation is a common mode of kinase regulation, acting to both positively and negatively modulate enzyme activity. Phosphorylation is required to turn on a number of protein kinases, including protein kinase A, and members of the mitogen-activated (MAP) and cyclin-dependent (cdk) protein kinase families. Interestingly, CKI is one of a small group of kinases that does not require such an activating catalytic domain phosphorylation. Phosphorylation also negatively regulates a substantial number of protein kinases. For example, phosphorylation of cyclin-dependent kinases on threonine and tyrosine residues in the ATP-binding loop by wee1-related ki-
nases leads to kinase inactivation, whereas phosphorylation of the Src tyrosine kinase by CRK on a tyrosine residue in the carboxyl terminus maintains the kinase in an inactive conformation (18). We and others have recently demonstrated that, in vitro, intramolecular autophosphorylation of the carboxyl-terminal domain also inhibits kinase activity of CKIε and CKIδ in a reversible manner. The present study indicates that this auto-inhibition occurs in vivo but is only detected when cellular protein phosphatases are inhibited. When the cellular phosphatases are active, kinase autophosphorylation is rapidly reversed.

The data suggest that the highly related kinases CKIε and CKIδ are both maintained in the dephosphorylated state in vivo by a futile cycle of autophosphorylation and rapid dephosphorylation. While the mAb raised against the carboxyl terminus of CKIε cross-reacts with CKIδ, we conclude that both kinases are subject to the same phosphorylation cycle. The data supporting this conclusion include the findings that CKIδ and CKIε antibodies identify similar but not identical bands, that endogenous and HA-tagged CKIδ undergoes an electrophoretic mobility shift in transfected cells after phosphatase inhibitor treatment, that CKIδ and CKIε actively autophosphorylate in vitro, and there is no remaining unshifted immunoreactive kinase after phosphatase inhibitor treatment, suggesting all of the endogenous CKIδ and CKIε can autophosphorylate.

These findings suggest that in vitro experiments with full-length CKIδ and CKIδ should be approached with caution since these kinases have the ability to rapidly autophosphorylate and autoinhibit. In cell extracts, the addition of phosphatase inhibitors leads to the autophosphorylation of these kinases even in the absence of added ATP. If the role of the dephosphorylated carboxyl terminus is substrate recognition or kinase targeting, then to study the kinase in vitro may necessitate mapping the inhibitory sites and producing mutant kinases that are unable to autoinhibit their activity. While it is inviting to attempt to determine CKI autophosphorylation sites on the basis of known kinase peptide preferences, analysis of the sequence of the carboxyl terminus of CKIδ and CKIδ show no signature CKI phosphorylation sites. While CKI prefers acidic residues upstream of the target serine or threonine, the iso-electric point of the tails is calculated to be near 12, with no runs of acidic residues next to potential phosphorylation sites. Since CKI can utilize phosphorylated residues as specificity determinants, it is possible that there is a directional step-wise phosphorylation of the tail, with each phosphorylation event creating a new target site.

What might perturb this regulation? CKIε and CKIδ have been implicated in DNA damage response pathways because of their homology to HRR25, the ability of CKIε to functionally complement HRR25-deficient yeast, and the ability of CKI isoforms to phosphorylate substrates such as p53, SV40 large T antigen, Swi6, and the 14–3–3 proteins. The finding that CKIδ and CKIδ are actively maintained in a hypophosphorylated state in both cultured cells and in tissues suggests that these kinases are maximally active under basal conditions. Previous studies have suggested that the activity of CKI on a target may be regulated by prior phosphorylation of that target by other kinases, creating a CKI recognition site (19). However, this mechanism does not appear to regulate phosphorylation of the p53, IkBα, ets-1, and 14–3–3 proteins, nor SV40 large T antigen (9, 10, 12, 20). Our results raise the additional possibility that CKIδ and CKIδ may also be regulated by specific intracellular phosphatases. One possible mode of regulation might then be changes in the phosphorylation state and hence the activity of the kinase, due to a change in the activity of the specific phosphatase that normally acts on the kinase. Decreased phosphatase activity would lead to increased CKI autophosphorylation and decreased CKI kinase activity, perhaps in response to DNA damage. Santos et al. (21) described a Drosophila melanogaster CKI isoform whose activity in immunoprecipitates was markedly stimulated by either alkaline phosphatase treatment or prior γ-irradiation of embryos, suggesting that this mechanism of CKI control may be widespread. While no specific modulators of global mammalian CKIε/δ phosphorylation state have yet been found, it may be that distinct intracellular subsets of the kinase will be regulated by locally active phosphatases. This mechanism could therefore differentially regulate CKI activity at different subcellular locations.

These studies suggest that one level of control of CKIδ and CKIδ activity is mediated by protein phosphatases. The change in CKIδ and CKIδ phosphorylation after treatment of cells with the phosphatase inhibitors okadaic acid or calyculin A suggests that either PP1 or PP2A-like phosphatases act on these kinases in vivo. Since the in vitro studies were performed with purified phosphatase catalytic subunits, we currently have no information on which phosphatase holoenzyme might be active on the kinase. The subcellular localization of these kinases suggests that nuclear as well as cytoplasmic forms of these phosphatases may be involved in this regulation. Multiple mechanisms to regulate these phosphatases have recently been elucidated, including phosphorylation (17, 22), polypeptide inhibitors including oncoproteins and developmental regulators (23, 24), and changes in phosphatase regulatory subunits (17, 22–25).

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REFERENCES
1. Manno, S., Takakuku, Y., Nagao, K., and Mohandas, N. (1995) J. Biol. Chem. 270, 5659–5665
2. Robinson, L. C., Menold, M. M., Garrett, S., and Culbertson, M. R. (1993) Mol. Cell. Biol. 13, 2870–2881
3. Hoekstra, M. F., Liskay, R. M., Ou, A. C., DeMaggio, A. J., Burbee, D. G., and Heffron, F. (1991) Science 253, 1031–1034
4. Dhillon, N., and Hoekstra, M. F. (1994) EMBO J. 13, 2777–2788
5. Knippschild, U., Milne, D. M., Campbell, L. E., DeMaggio, A. J., Christensson, E., Hoekstra, M. F., and Meek, D. W. (1997) Oncogene 15, 1727–1736
6. Graves, P. R., Haas, D. W., Hagedorn, C. H., DePaoli-Roach, A. A., Roach, P. J. (1993) J. Biol. Chem. 268, 6394–6401
7. Fish, K., Cegielska, A., Gietzen, K. F., Landes, G., and Virshup, D. M. (1995) J. Biol. Chem. 270, 14875–14883
8. Cegielska, A., and Virshup, D. M. (1995) Mol. Cell. Biol. 13, 1202–1211
9. Cegielska, A., Moarefi, I., Fanning, E., and Virshup, D. M. (1994) J. Viral. 68, 269–275
10. Cegielska, A., Gietzen, K. F., Rivers, A., and Virshup, D. M. (1998) J. Biol. Chem. 273, 1357–1364
11. Virshup, D. M., Russo, A., and Kelly, T. J. (1992) Mol. Cell. Biol. 12, 4883–4895
12. Dubois, T., Rommel, C., Howell, S., Steinshuss, U., Soneji, Y., Morrice, N., Moelling, K., and Atiken, A. (1997) J. Biol. Chem. 272, 28882–28888
13. Lin, R., Beauport, P., Makris, C., Meloche, S., and Hiscott, J. (1996) Mol. Cell. Biol. 16, 1401–1407
14. Ho, U. Mason, S., Kobayashi, R., Hoekstra, M. F., and Meek, D. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 581–586
15. Graves, P. R., and Roach, P. J. (1993) J. Biol. Chem. 270, 21689–21694
16. Wold, M. S., Weinberg, D. H., Virshup, D. M., Li, J. J., and Kelly, T. J. (1989) J. Biol. Chem. 264, 2801–2809
17. McCright, B., Rivers, A. M., Audlin, S., and Virshup, D. M. (1996) J. Biol. Chem. 271, 22081–22089
18. Xu, W., Harrison, S. C., and Eck, M. J. (1997) Nature 385, 595–602
19. Plotow, H., Graves, P. R., Wang, A. Q., Fiol, C. J., Rooske, R. W., and Roach, P. J. (1990) J. Biol. Chem. 265, 14264–14269
20. Meek, D. W., Campbell, L. E., Jardine, L. J., Knippschild, U., McIndrick, L., and Milne, D. M. (1997) Biochem. Soc. Trans. 25, 416–419
21. Santos, J., Logarinho, E., Tapia, C., Allende, C., Allende, J., and Sunkel, C. (1999) J. Cell Sci. 108, 1847–1856
22. Chen, J., Parsons, S., and Brautigan, D. L. (1994) J. Biol. Chem. 270, 581–586
23. Kawabe, T., Muslin, A. J., and Korsmeyer, S. J. (1997) Nature 385, 454–458
24. Li, M., Makkinje, A., and Damuni, Z. (1996) J. Biol. Chem. 271, 11059–11062
25. Cegielska, A., Shaffer, S., Derue, R., Gors, J., and Virshup, D. M. (1994) Mol. Cell. Biol. 14, 4616–4623