Autoinhibition of Casein Kinase I ε (CKIε) Is Relieved by Protein Phosphatases and Limited Proteolysis*

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Casein kinase I ε (CKIε) is a member of the CKI gene family, members of which are involved in the control of SV40 DNA replication, DNA repair, and cell metabolism. The mechanisms that regulate CKIε activity and substrate specificity are not well understood. We report that CKIε, which contains a highly phosphorylated 123-amino acid carboxy-terminal extension not present in CKIα, is substantially less active than CKIα in phosphorylating a number of substrates including SV40 large T antigen and is unable to inhibit the initiation of SV40 DNA replication. Two mechanisms for the activation of CKIε have been identified. First, limited tryptic digestion of CKIε produces a protease-resistant amino-terminal 39-kDa core kinase with several-fold enhanced activity. Second, phosphatase treatment of CKIε activates CKIε 5–20-fold toward T antigen. Similar treatment of a truncated form of CKIε produced only a 2-fold activation. Notably, this activation was transient; reautophosphorylation led to a rapid down-regulation of the kinase within 5 min. Phosphatase treatment also activated CKIε toward the novel substrates Iλα and Ets-1. These mechanisms may serve to regulate CKIε and related forms of CKI in the cell, perhaps in response to DNA damage.

The casein kinase I (CKI)1 gene family encompasses an increasing number of genes expressed in eukaryotes including yeast Caenorhabditis elegans and mammals. Two subgroups of the CKI family have been separated by functional analysis and complementation of mutations in yeast. One group encoding nuclear kinases appears in yeast to be involved in the response to DNA damage. Mutations in these genes, including YCK1 and YCK2, are involved in bud growth and includes YCK1 and YCK2 (6); deletions in these genes are complemented by the mammalian genes encoding CKIy (7).

The structure of the CKI family suggests several potential mechanisms for the regulation of activity. All family members contain a short amino-terminal domain of 9–76 amino acids, a highly conserved kinase domain of 284 amino acids, and a highly variable carboxy-terminal domain that ranges from 24 to more than 200 amino acids in length. The carboxy terminus of a CKI isoform may serve several functions, including regulation of substrate recognition, modulation of catalytic activity, and/or determination of kinase subcellular localization. Prenylation of the tail of the YCK1/YCK2 family has been shown to be of functional importance in yeast (3, 8, 9). Phosphorylation of CKI may also be an important regulatory mechanism. Most of the CKI proteins are phosphoproteins, and several of the yeast kinases can autophosphorylate on serine, threonine, and tyrosine residues (10). Studies using synthetic substrates and the artificial substrate casein have indicated that these phosphorylation residues may inhibit kinase activity (11, 12), although the location of these inhibitory groups remains unclear. Kuret and co-workers (11) found that an unphosphorylated truncation of CKI containing only the kinase domain was twice as active as the phosphorylated form, whereas Graves et al. (12) mapped a phosphorylation-dependent inhibitory domain in CKIα to a 26-amino acid domain in the carboxy-terminal tail. Polyamines such as spermine can regulate kinase activity; activation by spermine appears dependent on the presence or absence of the carboxy-terminal domain (12, 13). Additionally, membrane bound forms of the kinase may be regulated by phosphatidylinositol-4,5-bisphosphate (14).

One in vitro functional assay of CKI activity is its ability to phosphorylate simian virus 40 (SV40) large T antigen. We have previously shown that CKIε purified from HeLa cell extracts phosphorylates T antigen on physiologic sites and inhibits the initiation of viral DNA replication (15–17). As SV40 DNA replication is regulated in the cell cycle and by DNA damage (18–21), it was of interest to determine whether forms of CKI implicated in DNA repair pathways could also regulate in vitro DNA replication.

We have now overexpressed and purified active CKIε. The data show that this form of CKI, although active on peptide substrates, is markedly hindered in its ability to phosphorylate and inhibit the origin unwinding function of T antigen. This decreased activity of CKIε is apparently due to an inhibitory effect of the carboxy-terminal domain not present in CKIα, since limited tryptic digestion of CKIε released a catalytically active amino-terminal 39-kDa fragment able to both phospho-
rylate T antigen and inhibit its replication initiation function.

We also report that CKIε is activated by dephosphorylation in a tail-dependent manner. Treatment of recombinant CKIε with the catalytic subunits of PP1, PP2A, or PP2B (calcineurin) leads to as much as a 20-fold increase in activity toward T antigen, casein, and two novel substrates, the Ets-1 transcription factor and recombinant IκBα. Activation of the kinase by phosphatases was transient and self-limited; reautophosphorylation of the kinase led to inactivation within 5 min. Activation was dependent on the presence of the carboxyl-terminal domain of the kinase; a truncation mutant of CKIε was activated by phosphatase 4-fold less than was full-length kinase. These findings support the hypothesis that the carboxyl-terminal domain of CKIε inhibits its activity on key protein substrates and suggests that in the cell, CKIε may be regulated in a self-limited manner by phosphatases and in a more sustained manner by intracellular proteolysis.

MATERIALS AND METHODS

NF²⁺-nitrotriacetate-agarose was obtained from Qiagen. Trypsin (T8642), calcineurin, and calmodulin were from Sigma. PP1, and inhibitor II were from New England Biolabs. Okadaic acid was from Life Technologies, Inc. Restriction enzymes were from Life Technologies, Inc. and New England Biolabs. Plasmids expressing IκBα (22) were the generous gift of John Hiscott.

Cloning and Escherichia coli Expression of CKIε—The cdNA encoding wild type human CKIε was isolated as a Ncol/SalI 1333-base pair fragment and ligated into Ncol/XhoI-digested pET16b (a T7-based expression vector from Novagen) as described previously (35). This construct (pKF115) removes the hexahistidine sequence present in the pET16b vector downstream from the Ncol site. The 1317-base pair Ncol/HindIII fragment from pKF115 was ligated into the same sites in pRSET-B (a T7-based expression vector from Invitrogen). This construct (pV71) encodes CKIε with a 41-amino acid amino-terminal fusion that contains a hexahistidine tag and an enterokinase cleavage site. The d305 truncation mutant was created by the introduction of a stop codon after amino acid 305 in a derivative of pV71 (pKF162) by site-directed mutagenesis. All recombinant proteins were expressed in BL21(DE3) cells (23). Bacteria were grown in Luria broth containing 50 μg/ml carbenicillin at 37 °C to an A600 of 0.3 and induced overnight at 28 °C with 1 mM isopropyl-1-thio-β-D-galactopyranoside.

Purification and Partial Proteolysis of Recombinant CKIε—Hexahistidine-tagged CKIε was expressed at low levels in BL21(DE3) cells. Lysates were present in 20 mM Hepes, pH 7.5, 25 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.02% Nonidet P-40, 10% sucrose (buffer B) with 0.1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin were applied to DEAE-cellulose and batch-eluted with the same buffer containing 200 mM NaCl. Kinase-containing fractions were dialyzed into buffer containing 10 mM NaCl and then applied to a S-Sepharose column equilibrated with the same buffer. Kinase activity was batch-eluted with buffer B with 250 mM NaCl (without EDTA or DTT) and loaded directly onto Ni²⁺-nitrotriacetate-agarose from which it was eluted with buffer B (without EDTA or DTT) containing 80 mM imidazole. Untagged CKIε was purified by a similar procedure except that the Ni²⁺-nitrotriacetate-agarose step was omitted. The typical yield was 100 μg of CKIε from a 2-liter culture. Hexahistidine-tagged CKIε was used in all assays except where specifically indicated. Histidine-tagged and untagged kinase were found to behave identically in all assays tested.

Proteolysis of purified wild type and hexahistidine-tagged CKIε was performed in the standard kinase reaction buffer (30 mM Hepes, pH 7.5, 7 mM MgCl₂, 0.5 mM DTT for 30 °C for 15 min either without or with previous autophosphorylation of substrate. Trypsin was then inhibited by soybean trypsin inhibitor added to a final concentration of 10 μg/ml. Proteolysis with trypsin at 1 μg/ml gave almost quantitative scission of CKIε into its major digestion products; this concentration of trypsin was routinely used for the production of the active tryptic fragment.

Immunoblot Analysis—For immunoblot analysis, proteins and trypsin-generated peptides were separated by SDS-PAGE and autoradiography on a 17% gel and then transferred to nitrocellulose membrane in 12.5 mM Tris, 88 mM glycine, pH 8.3, 0.1% SDS, 20% methanol. After a 15-min fixation in 0.5% glutaraldehyde in phosphate-buffered saline, the membrane was blocked with 3% bovine serum albumin and then incubated with a 1:500 dilution of UT31 antiserum raised against the amino-terminal CKIε peptide MELRVGNYRLGC (5). Immunoreactive peptides were detected using an alkaline phosphatase-conjugated goat-anti-rabbit IgG (Bio-Rad) followed by incubation with bromo-chloro-indolylphosphate and nitroblue tetrazolium (24).

Kinase and Phosphatase Assays—Kinase reactions were performed in buffer containing 30 mM Hepes, pH 7.5, 7 mM MgCl₂, 0.5 mM DTT, 100 μM bovine serum albumin, 150 or 250 μM ATP, and 1–5 μCi of [γ-³²P]ATP at 37 °C or as indicated. Reactions were stopped by the addition of SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography as described previously (15, 16).

Peptide phosphorylation reactions contained the synthetic 15-mer phosphopeptide substrate AHALS(P)VSALPGLKKK (termed 5P) that was synthesized with the serine in position 5 phosphorylated. This peptide contains a CKI consensus site and is phosphorylated by both CKIα and CKIε with a Kₗ of approximately 200 μM.²

Peptide kinase assays were terminated by the addition of 50 μl of 30% acetic acid/10 μl assay and quantitated by spotting the reaction mixture onto P81 phosphocellulose filters as described (25). All phosphatase reactions were performed in 30 mM Hepes, pH 7.5, 7 mM MgCl₂, and 100 μg/ml bovine serum albumin unless otherwise noted. Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (26).

RESULTS

Substrate Specificity—In previous studies we purified CKIα based on its ability to phosphorylate SV40 large T antigen and thus inhibit the initiation of SV40 DNA replication (15, 16). In the process of characterizing human CKI genes, we cloned a novel member of the CKI family, CKIε, that encodes a 48-kDa kinase (5). CKIε was of interest because, unlike CKIα, it was able to functionally complement yeast deleted for the DNA repair-related kinase HRR25. In preliminary studies on recombinant CKIε, we found virtually identical specific activity to CKIα when tested on peptide substrates (data not shown). However, although CKIε was found to readily phosphorylate T antigen, CKIε was approximately 9-fold less active on this protein substrate (Fig. 1). Similar results were obtained when a partially purified non-tagged form of CKIε was tested (data not shown), indicating the histidine tag has no significant effect on the ability of CKIε to phosphorylate T antigen. Interestingly, CKIε autophosphorylation was also consistently stimulated severalfold by the addition of T antigen (Fig. 1, lane 4 compared with lanes 5 and 6), suggesting a specific protein-protein interaction between the carboxyl-terminal domain and the substrate.

² A. Cegielska, E. Vielhaber and D. M. Virshup, unpublished results.
Effect of Carboxyl-terminal Tail on CKIe Activity—CKIe and CKIa are closely related in the kinase domain (86% similar and 74% identical) but differ in the length of the carboxyl-terminal tail. CKIe has only a 24-amino acid extension beyond the kinase domain, whereas CKIa has a 123-amino acid tail. Recent studies of carboxyl-terminal domains in the related kinases CKI8 and Ckl1 have indicated that they perform a regulatory function (11, 27). The differing activities of the CKIe and CKIa isoforms suggested that the carboxyl-terminal domain of CKIe was responsible for the decreased activity of the kinase on T antigen and secondly, that this region might form a discrete structure separable from the kinase domain by limited proteolysis. To test this, intact autophosphorylated CKIe was subjected to limited digestion with various concentrations (0–5 μg/ml) of trypsin. As demonstrated in Fig. 2, digestion of 32P-autophosphorylated CKIe with increasing amounts of trypsin results in the production of a series of higher mobility species that are further digested to a relatively stable 39-kDa phosphoprotein that finally disappears during incubation at the highest trypsin concentration (5 μg/ml). This 39-kDa product appears to be activated CKIe since (a) its appearance coincided with a 3-fold increase in kinase activity toward T antigen, (b) it reacts with polyclonal antibody UT31 that recognizes the amino terminus of CKIe, and (c) the purified 39-kDa polypeptide retains kinase activity (Fig. 3 and data not shown). The 3-fold activation achieved by trypsin treatment may under-represent the actual activation since (a) there may have been partial loss of the kinase domain as well, and (b) proteolytic tail fragments may retain some inhibitory activity (data not shown). These results suggest that CKIe contains a discrete inhibitory domain that blocks specific substrate phosphorylation.

Functional Activity of the 39-kDa Amino-terminal Fragment of CKIe Protein—T antigen catalyzes the initial steps in SV40 DNA replication, the unwinding of the duplex origin of replication to single-stranded DNA. CKIa inhibits T antigen function by phosphorylating it on at least two inhibitory sites, serines 120 and 123 (15, 16). The finding that proteolytic cleavage of CKIa stimulated its activity on T antigen led to the further functional characterization of the 39-kDa tryptic fragment. Specifically, we wished to determine whether its increased protein kinase activity translated into an increased ability to inhibit T antigen-catalyzed SV40 origin unwinding. CKIe, CKIa, and the 39-kDa CKIe fragment (CKIe-39) were pre-incubated with T antigen and ATP, and then T antigen-dependent unwinding of the SV40 minimal origin of replication was assayed (Fig. 3). As previously demonstrated, phosphorylation of T antigen by CKIa inhibited its origin unwinding activity with 50% inhibition occurring at about 0.4 pmol of kinase/reaction (40 nM in the preincubation mixture). In contrast, CKIe very inefficiently inhibited the origin unwinding activity of T antigen with 50% inhibition occurring at about 7 pmol of kinase/reaction (700 nM in preincubation mixture). The 39-kDa fragment of CKIe was substantially more active than full-length CKIe, producing 50% inhibition of T antigen origin unwinding activity at about 0.9 pmol of kinase/reaction (90 nM in preincubation mixture, Fig. 3B). These results indicate that the catalytic core of CKIe is in fact similar in activity to CKIa and that proteolytic removal of the carboxyl terminus of CKIe partially activates it to phosphorylate the same or similar inhibitory sites on T antigen.

Diverse Serine/Threonine Phosphatases Activate CKIe toward T Antigen—Recent studies on the related CKI isoform CKI8 have demonstrated that the full-length kinase can be activated 2–5-fold toward casein or a synthetic peptide (D4) by treatment with the catalytic subunit of protein phosphatase 1 (PP1c) (27). We tested whether removal of phosphoryl groups resulted in partial activation of CKIe toward T antigen in an assay similar to that described above. As demonstrated in Fig. 3, these Diverse Serine/Threonine Phosphatases Activate CKIe toward T Antigen—Recent studies on the related CKI isoform CKI8 have demonstrated that the full-length kinase can be activated 2–5-fold toward casein or a synthetic peptide (D4) by treatment with the catalytic subunit of protein phosphatase 1 (PP1c) (27). We tested whether removal of phosphoryl groups...
addition of 250 nM okadaic acid, and kinase activity was assessed by the

After the preincubation, phosphatase activity was inhibited by the

approximately 12 mol/mol. CKI was dephosphorylated with PP2A, and then allowed to reautophosphorylate in the presence of okadaic acid. T antigen was either added at the same time as [γ-32P]ATP (time 0) or at the indicated times after ATP addition. As Fig. 5 demonstrates, CKI reautophosphorylation was complete within 5 min, coincident with the re-suppression of kinase activity. Thus, CKI appears to re-autophosphorylate and autoinhibit itself rapidly in the absence of phosphatase activity. Dilution studies and experiments with CKI kinase dead mutants indicate that CKI autophosphorylation is entirely intramolecular (data not shown). Of note, neither activation nor autoinhibition of kinase activity toward a peptide substrate was seen in similar time course reactions (data not shown), suggesting autoinhibition is effective toward protein but not peptide substrates.

Bacterially-produced CKI is Heavily Autophosphorylated—Dephosphorylation of bacterially expressed CKI had a significant activating effect on the kinase. To determine the number of phosphates on CKI that contribute to kinase inhibition, CKI autophosphorylation was quantitated after pretreatment with increasing amounts of PP2A, or PP1, (Fig. 4C). Untreated kinase was able to add about 2.5 mol of phosphate/mol of kinase, whereas PP2A-treated kinase added about 12 mol/mol. These data suggest that the kinase was close to maximally autophosphorylated at approximately 10 mol/mol when it was purified from E. coli. Half-maximal activation of the kinase toward T antigen was seen when the kinase added approximately 8 mol/mol, suggesting that 4 mol/mol of phosphate had not been removed by the pretreatment. Similar results were seen with PP1.

Phosphoamino acid analysis performed on CKI pretreated with PP2A, and autophosphorylated in the presence of [γ-32P]ATP indicates that autophosphorylation occurs on both serine and threonine residues (data not shown). Although CKI family members have been shown to be able to autophosphorylate on tyrosine as well as on serine and threonine (10), the autophosphorylation experiments in this study address only serine and threonine phosphorylation as the phosphatases used are serine/threonine-specific. The data indicate that removal of phosphoryl groups from serine and threonine but not tyrosine residues is responsible for the activation of the kinase.

PP2A Activates CKI toward an Array of Protein Substrates—To determine whether dephosphorylation of CKI activated it toward multiple protein substrates, the kinase was

from CKI stimulated its kinase activity toward SV40 large T antigen (Fig. 4, A and B). Purified CKI was incubated with increasing amounts of the catalytic subunit of either PP1 or PP2A. At the end of the dephosphorylation reaction, okadaic acid was added to a final concentration of 250 nM, and the activity of the kinase on T antigen was assessed. As demonstrated in Fig. 4, A and B, pretreatment of CKI with either phosphatase stimulates its activity on T antigen up to 20-fold, with half-maximal activation occurring with less than 2 ng (about 4 nM) of PP2A. The stimulation requires phosphatase catalytic activity, since inclusion of okadaic acid in the dephosphorylation fully blocks the effect of PP2A (Fig. 4A, lane 7). The activation by 16 ng (about 30 nM) of PP1 is only partially blocked by 250 nM okadaic acid, a not unexpected result since PP1 is 10-100-fold less sensitive than PP2A, to inhibition by okadaic acid. Of note, alterations in the phosphorylation state of CKI had a significant effect on kinase mobility (Fig. 4A). This appears to be due to tail phosphorylation, as truncated CKI can autophosphorylate without a significant change in electrophoretic mobility (Fig. 7A and data not shown).

Autophosphorylation Rapidly Inactivates CKI—CKI is capable of autophosphorylation of up to approximately 12 mol/mol (Fig. 4C and see below). Since phosphatase treatment of the bacterially expressed kinase leads to marked activation toward protein substrates, we asked how rapidly autophosphorylation of the kinase occurs and whether this autophosphorylation correlates with a decrease in protein kinase activity. CKI was dephosphorylated with PP2A, and then allowed to reautophosphorylate in the presence of okadaic acid. T antigen was either added at the same time as [γ-32P]ATP (time 0) or at the indicated times after ATP addition. As Fig. 5 demonstrates, CKI reautophosphorylation was complete within 5 min, coincident with the re-suppression of kinase activity. Thus, CKI appears to re-autophosphorylate and autoinhibit itself rapidly in the absence of phosphatase activity. Dilution studies and experiments with CKI kinase dead mutants indicate that CKI autophosphorylation is entirely intramolecular (data not shown). Of note, neither activation nor autoinhibition of kinase activity toward a peptide substrate was seen in similar time course reactions (data not shown), suggesting autoinhibition is effective toward protein but not peptide substrates.
tested using casein, replication protein A (the gift of Marc Wold, U. Iowa), and recombinant Ets-1 (the gift of Barbara Graves, U. Utah) as substrates. As demonstrated in Fig. 6A, CKI is activated toward T antigen, casein, and Ets-1 but not replication protein A (RP-A) by PP2A, treatment. Similarly, treatment of CKI with PP1, PP2A, or PP2B catalytic subunit activated the kinase toward GST-IκBa (Fig. 6B). The activation of CKI by phosphatases is therefore a general phenomenon. Whereas CKI substrate specificity on peptides can be determined by phosphoryl groups, we note that the increase in kinase activity occurs toward unphosphorylated (bacterially produced Ets-1 and GST-IκBa) as well as phosphorylated (T antigen) proteins. 

**CKI Phosphorylates the Carboxyl Terminus of IκBa**—Although genetic studies have suggested a role for CKI-related proteins in DNA damage response pathways, few physiologic substrates of CKI have been identified. As IκBa is phosphorylated and degraded in response to many signals including DNA damage and it contains sequences in the amino and carboxyl termini similar to CKI phosphorylation consensus sites found in peptide substrates, we tested whether CKI could be activated to phosphorylate IκBa. As shown in Fig. 6B, CKI phosphorylates GST-IκBa in a phosphatase-activatable manner.

**Fig. 5. Rapid reautophosphorylation of CKIe leads to inhibition of kinase activity within 5 min.** A and B, CKIe was preincubated alone (lane 1) or activated by preincubation with 4 ng of PP2A, (lanes 2–6). At the end of the preincubation, okadaic acid (OA) and [γ-32P]ATP were added to all reactions to final concentrations of 250 nM and 100 μM, respectively. T antigen (T ag) was added either at the end of the preincubation (time 0, lanes 1 and 2) or at the indicated times after ATP and okadaic acid (OA) addition (lanes 3–6). The T antigen kinase reaction proceeded for 15 min. T antigen phosphorylation was quantitated by SDS-PAGE and PhosphorImager analysis. A, autoradiograph of the kinase reaction. T antigen is indicated by an open circle, and duration of the autophosphorylation reaction (min) is denoted at the top of each lane. B, quantitation of T antigen phosphorylation. CKIe activity toward T antigen was activated by PP2A, but was markedly diminished by a 5-min autophosphorylation reaction with ATP and okadaic acid.

**Fig. 6. Dephosphorylation activates CKIe toward diverse substrates.** A, CKIe is activated toward T antigen, casein, and Ets-1. CKIe (125 ng) was preincubated without (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and 8) 4 ng of PP2A, and then incubated with okadaic acid, 100 μM [γ-32P]ATP, and either 0.24 μg of T antigen (T, 90 kDa, lanes 1 and 2), 1 μg of casein (C, 27 kDa, lanes 3 and 4), 1 μg of replication protein A (RP-A) (R1, 70 kDa, lanes 5 and 7) and 1 μg of recombinant Ets-1 (E, 50 kDa, lanes 7 and 8). An open circle denotes autophosphorylated CKIe. B, CKIe is activated toward IκBa by PP2A, PP1, and PP2B. CKIe (20 ng) was preincubated alone (lanes 1, 3, and 5) or with PP2A (0 ng, lane 2), PP1 (0.5 units, lane 4), or calcineurin (10 units, lane 6) for 15 min at 37 °C in buffer containing 7 mM MgCl₂, 30 mM Hepes, pH 7.5, 100 μg/ml bovine serum albumin, 100 μM CaCl₂, and 2 units of calmodulin. After the preincubation, kinase activity was assayed for 5 min at 37 °C in the presence of 3 μg of GST-IκBa and 100 μM [γ-32P]ATP with 100 ng of inhibitor 2,250 nM okadaic acid, and 1 mM EGTA. Reactants were separated by 10% SDS-PAGE and analyzed by PhosphorImager. The expected migration of GST-IκBa is indicated by an arrow and that of CKIe is indicated by an open circle. C, carboxyl-terminal truncation mutants of IκBa are not substrates for CKIe. PP2A- treated CKIe was incubated with 400 ng of wildtype or carboxyl-terminal truncation forms of GST-IκBa for 5 min at 37 °C. Reactants were separated by 8% SDS-PAGE, silver-stained, and analyzed by PhosphorImager. WT, full-length GST-IκBa; ΔE, deletion of amino acids 369–317; ΔA, deletion of amino acids 288–317 (nomenclature of Ref. 22). Removal of the terminal 22 amino acids (mutant ΔA) was sufficient to abrogate CKI phosphorylation of IκBa, suggesting carboxyl-terminal structure is essential for kinase recognition of the substrate. autorad, autoradiography.

The serine/threonine phosphatases PP2A, PP1, and PP2B/calcineurin were all able to activate CKIe to phosphorylate recombinant IκBa. Two regions of phosphorylation of IκBa have been identified; serines 32 and 36 in the amino terminus are essential for the ubiquitin-mediated degradation of IκBa bound to NF-κB, whereas phosphorylation of extreme carboxyl-terminal residues in a PEST region regulates proteolysis of free IκBa (22, 28, 29). To determine whether CKI phosphorylated the carboxyl terminus of IκBa, the ability of activated kinase to phosphorylate full-length and carboxyl-terminal truncation mutants (the generous gift of John Hiscott) was assayed. As shown in Fig. 6C, CKIe phosphorylated full-length GST-IκBa (lanes 1 and 5) but had no activity on mutants lacking the carboxyl-
terminal 22 residues or greater (lanes 2–4, 6–8) (22). Note that
although CKIε phosphorylates IxBα on both serine and threo-
ine (data not shown), the nonphosphorylated Δ4 truncation
removes two threonine but no serine residues. Studies with
point mutants suggest serines 288 and 293 are phosphorylated
by CKIε (data not shown). The Δ4 mutant (truncated at amino
cid 296) has been shown to be a substrate for the unrelated
kinase, casein kinase II, indicating that the truncation does not
lead to denatured protein (22). The data therefore suggest that
removal of the carboxyl-terminal 22 amino acids disrupts the
local structure enough to interfere with CKI but not CKII
activity on IxBα. These results are consistent with our previous
data (15) indicating that CKI activity on protein (but not pep-
tide) substrates is highly dependent on the intact tertiary
structure of the substrate.

Full Activation by Phosphatases Requires the Carboxyl Ter-
ninus of CKIε—One model to explain both the inhibitory effect
of the carboxyl-terminal tail and the stimulatory effect of de-
phosphorylation is that phosphate groups on the carboxyl ter-
ninus of CKIε interact with the kinase domain, leading to
inhibition of protein substrate binding. This model suggests
that a tail-less CKIε should not be activated by phosphatases.
To test this, a stop codon was introduced after amino acid 305
in CKIε, and the truncated histidine-tagged protein (denoted
CKIε-d305) was expressed in E. coli and purified on Ni²⁺-
nitrilotriacetate-agarose. Roughly equal amounts of full-length
and truncated CKIε were used to phosphorylate T antigen,
either without or with prior phosphatase treatment. As shown
in Fig. 7, CKIε was activated 9-fold by PP2Aα, whereas CKIε-
d305 was activated only 2-fold. Similarly, CKIε (76% identical
to CKIε over the kinase domain and lacking a carboxyl-termi-
nal tail) was not activated by PP2Aα (data not shown). The data
suggest that much of the observed autoinhibition of CKIε
requires the carboxyl-terminal tail, but that an inhibitory phos-
phorylation group on the CKIε kinase domain also contributes to
autoinhibition. These results are consistent with those seen by
Kuret and co-workers (11) on the S. pombe homolog Cki1-d298
but differ slightly from those of Graves and Roach (27) who
found that truncated CKIδ was not activated by phosphatase
treatment.

Previous work demonstrated that the activity of CKIε toward
peptide substrates was not significantly influenced by the pres-
ence of the carboxyl-terminal domain. We next tested whether
phosphatase activation of the kinases had an effect on activity
toward peptide, as opposed to protein, substrates. As shown in
Fig. 7C, PP2Aα selectively activated CKIε toward T antigen but
not the 5P peptide substrate.

**DISCUSSION**

The casein kinase I family is characterized by a conserved
core kinase domain and a series of variable carboxyl-terminal
extensions. This study indicates that one major function of the
carboxyl terminus is to regulate the activity of CKI on protein
substrates. The ability of the carboxyl terminus to inhibit pro-
tein but not peptide phosphorylation suggests that this tail
region interacts with the substrate binding face of the kinase,
as illustrated in Fig. 8, rather than directly in the catalytic
cleft. The data suggest CKI autoinhibition may be relieved by
at least three mechanisms: (a) proteolytic cleavage of the tail
(this study), (b) dephosphorylation of the kinase (this study and
Refs. 11 and 27), and (c) binding of heparin (and presumably
other polyanions) to the tail (27). Which, if any, of these me-
chanisms function in vivo is the subject of ongoing investigation.
One reason for the diversity in CKI carboxyl-terminal domains
may be to allow distinct activation mechanisms for the differ-
ent family members. Alternatively, the function of the tail may
be to constitutively restrict access to the catalytic cleft to all but

![Fig. 7](image)

**Fig. 7. The carboxyl terminus of CKIε is the major determi-
nant of PP2Aα-mediated activation.** A and B, PP2Aα, activated CKIε
9.1-fold and CKIε-d305 2.0-fold toward T antigen. 85 ng each of CKIε
(lanes 1 and 2) or CKIε-d305 (lanes 3 and 4) lacking the carboxyl-
terminal 111 amino acids of CKIε were incubated without (lanes 1 and
3) or with (lanes 2 and 4) 8 ng of PP2Aα for 15 min at 37 °C before the
addition of okadaic acid, T antigen, and [γ-32P]ATP for a 3-min kinase
reaction. Reaction products were separated by SDS-PAGE and quanti-
tated by PhosphorImager analysis. T antigen is indicated by an arrow,
and CKIε and CKIε-d305 are indicated by open circles. A, autoradi-
ograph; B, PhosphorImager quantitation. C, comparison of activation
of CKIε by PP2Aα toward T antigen (closed circles, solid line) and the 5P
peptide (open circles, dashed line). The kinase reactions were identical
to those above but lasted only 2 min.

a limited number of substrates, and no further regulation of
the kinase may occur in vivo. Differentiation between these me-
chanisms will require the demonstration and characterization
of CKI activation in vivo. Given the functional similarity between
CKIε and HRR25, one possibility is that agents that trigger the
DNA repair response will lead to CKIε activation.
Activation of forms of CKI lacking a carboxyl-terminal extension by dephosphorylation suggests that at least one inhibitory phosphoryl group is present on the kinase domain itself. Cleavage of CKI by regulated intracellular proteolysis would lead to a long-lived activation of CKI in response to the appropriate stimulus. Proteolytic activation has been demonstrated for an increasing number of intracellular proteins, including protein kinase C, mitogen-activated protein kinase kinase kinase 1 (MEKK1), and NF-\(\kappa\)B (31–33). DNA damage resulting in protease activation might lead to CKI cleavage. Notably, caspases with DEVD sequence specificity (34) may be activated during apoptosis and cleave in the acidic region of CKI at the beginning of the carboxyl terminus. Such cleavage might either activate CKI or release it from its normal anchoring site.

Previous studies have suggested that CKI recognizes peptide and protein substrates differently. CKI phosphorylates acidic peptides, with the best peptide substrates containing either phosphorylated or acidic residues amino-terminal to the target site (35, 36). In this case, peptide recognition requires interaction of the phosphorylated region with the catalytic fold (37, 38). However, this localized charge interaction does not appear operative in the case of T antigen phosphorylation. We have previously shown that (a) CKI can phosphorylate bacterially expressed (and hence unphosphorylated) T antigen and (b) CKI does not recognize its target site in the amino terminus of T antigen unless the carboxyl-terminal residues of T antigen are present (15). Thus, CKI could not phosphorylate a T antigen-derived peptide, an amino-terminal tryptic fragment of T antigen, nor T259, an active recombinant amino-terminal fragment of T antigen that contains the target sites and the DNA binding domain. Similarly, in the current study we found that removal of the carboxyl terminus of IxBo blocks phosphorylation of upstream residues. These results are consistent with a more complex picture of kinase-substrate interactions, where the tertiary structure of the substrate interacts with multiple surface features of the kinase. Therefore, additional structural elements of the kinase may interfere with docking of substrate proteins at sites distant from the target residues. One function of the CKI tail may be to restrict the access of protein substrates to the active site until the kinase is activated by transient dephosphorylation or limited proteolysis.

However, these results do not exclude the possibility that in vitro, the inhibitory effect of the tail observed in vitro could also be overcome by constitutive active dephosphorylation or additional mechanisms such as noncovalent binding of regulatory molecules.

Identification of potential cellular substrates of CKI has been problematic. CKI isoforms can phosphorylate SV40 large T antigen (15, 16), p53 (39), inhibitor-2 (30), and glycogen synthase, among others. The present study extends the list by identifying the carboxyl terminus of IxBo as an in vitro substrate. Whether CKI is as important as CKII in the phosphorylation of IxBo in vivo is unclear. In very few cases there is a clear functional role for the mammalian CKI family been defined. One approach to this question has been to overexpress kinases in cells or organisms and examine them for changes in phosphorylation patterns. An implication of this current study is that overexpression of CKI and related CKI family members may produce inactive, autoinhibited kinase with no phenotype or effect on in vivo substrates unless point mutations, truncations, or activating conditions are first introduced. One possible route to the identification of additional in vivo-specific substrates of CKI is therefore the development of autophosphorylation site point mutants that retain an intact regulatory domain but are constitutively active.

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