Identification of Inhibitory Autophosphorylation Sites in Casein Kinase I ε*

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Casein kinase I ε (CKIε) is a widely expressed protein kinase implicated in the regulation of diverse cellular processes including DNA replication and repair, nuclear trafficking, and circadian rhythm. CKIε and the closely related CKIβ are regulated in part through autophosphorylation of their carboxyl-terminal extensions, resulting in down-regulation of enzyme activity. Treatment of CKIε with any of several serine/threonine phosphatases causes a marked increase in kinase activity that is self-limited. To identify the sites of inhibitory autophosphorylation, a series of carboxyl-terminal deletion mutants was constructed by site-directed mutagenesis. Truncations that eliminated specific phosphopeptides in the wild-type kinase were used to guide construction of specific serine/threonine to alanine mutants. Amino acids Ser-323, Thr-325, Thr-334, Thr-337, Ser-368, Ser-405, Thr-407, and Ser-408 in the carboxyl-terminal tail of CKIε were identified as probable in vivo autophosphorylation sites. A recombinant CKIε protein with serine and threonine to alanine mutations eliminating these autophosphorylation sites was 8-fold more active than wild-type CKIε using 16B6 as a substrate. The identified autophosphorylation sites do not conform to CKI substrate motifs identified in peptide substrates.

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* The abbreviations used are: CKI, casein kinase I; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) kinase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PP2A, protein phosphatase 2A catalytic subunit.

terminal phosphorylation, whereas HRR25 is predominantly nuclear (8–10). CKI isoforms have also been identified in the cytosol and the nucleus and on mitotic spindles (11–13).

An increasing number of potential physiologic substrates of casein kinase I isoforms have been identified, but how the activity of the CKI family members on those substrates is regulated is generally not known. CKI isoforms in vitro preferentially phosphorylate peptides with acidic or phosphorylated residues N-terminal of the target site (14, 15), and therefore prior phosphorylation of the substrate is one potential mechanism for regulation of kinase activity. CKIε and the related kinase CKIβ phosphorylate N-terminal residues of p53 in vitro and in vivo (16, 17); this activity is enhanced by DNA damaging drugs. CKIα binding to NF-AT4 may be regulated by MEKK1 (7), whereas a CKIα homolog in Drosophila has been reported to change subcellular localization and activity in response to irradiation (18).

One way the activity, localization, and specificity of CKI isoforms may be regulated is through their diverse carboxyl-terminal domains. CKI family members have a similar primary sequence arrangement consisting of a highly conserved amino-terminal catalytic domain of approximately 283 amino acids and carboxyl-terminal extensions of variable length and sequence. Interestingly, although the kinase domains are highly conserved between species (e.g. human CKIα and yeast HRR25 kinase domains are 64% identical and 81% similar), the carboxyl termini in general have no discernible sequence homology. One exception to this is in mammals, where the 124-amino acid tail of CKIε is 50% identical to the tail of CKIβ. Several lines of evidence suggest the activities of CKIβ and CKIε are regulated by a carboxyl-terminal phosphorylation-dependent autoinhibitory domain (19, 20). Autophosphorylation both inactivates the kinase and leads to the accumulation of up to 8 mol of phosphate/mol of kinase. Removal of the CKIβ or CKIε carboxyl-terminal domain by mutagenesis or proteolysis reactivates the kinases. Furthermore, Graves and Roach (20) showed that transfer of the CKIβ tail to CKIε conferred autoinhibition on that chimeric kinase as well. Interestingly, in vivo, these kinases also autophosphorylate, but this autophosphorylation is rapidly reversed by endogenous protein phosphatases in a futile autophosphorylation-dephosphorylation cycle (13). The specific function of this futile cycle is not known, but it is potentially a mechanism to regulate either kinase activity or the ability of specific substrates to bind to the kinase.

To further study the function of CKIε in vitro and in vivo, we mapped the regulatory autophosphorylation sites on the CKIε carboxyl terminus. Progressive truncation of CKIε eliminated both its autophosphorylation sites and the ability to activate the kinase by dephosphorylating it with protein phosphatase 2A. Potential phosphorylation sites were then identified by two-dimensional phosphopeptide mapping. Mutation of specific
residues to alanine produced a recombinant enzyme with 8-fold higher specific activity. Interestingly, none of the identified CKI autophosphorylation sites conform to the consensus sites determined by studies of synthetic peptides.

MATERIALS AND METHODS

Ni²⁺-nitrotetrazolium-agarose was obtained from Qiagen. Trypsin (T6842) and cellulose plates were from Sigma. Okadaic acid and calyculin A were from Life Technologies, Inc. and CalBiochem, respectively. Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were from Life Technologies, Inc. and New England Biolabs. Anti-CKI monoclonal antibody was from Transduction Laboratories. Primers and peptides were obtained from the DNA/Peptide Facility at the University of Utah. An expression construct for CKIα was the gift of Paul Graves and Peter Roach, and purified CKIα protein was graciously provided by Erica Vielhaber.

Metabolic Labeling and Mapping of in Vivo Phosphorylation Sites—

The human embryonic kidney cell line 293 was transiently transfected with cytomegalovirus expression constructs pKFI82 or pKFI83 (19) or with empty vector (pCEP4-lerner). Cells at approximately 80% confluence were transfected with 2 ml of plasmid DNA modified by replacing a 1-kilobase pair fragment bordered by NdeI sites. The PCR product was digested with NdeI and replaced by a similar fragment from pAlter-1 (Promega) containing a nonfunctional ampicillin resistance gene. The pKF158 plasmid was used for both site-directed mutagenesis and overexpression in Escherichia coli.

Site-directed Mutagenesis of CKIα—Site-directed mutagenesis was conducted essentially by the Altered Sites method (Promega). Individual primary amp' transformants of E. coli strain 71–18 mutS were screened for either the presence or absence of the restriction site introduced or eliminated with each mutation (see Table I).

Expression and Partial Purification of CKIα—Recombinant histidine-tagged CKIα proteins (wild type and mutants) were expressed in BL21(DE3) cells containing the pphys plasmid (21, 22) as described previously (19). Clarified lysates in 30 mM HEPES, pH 7.5, 500 mM NaCl, 0.02% Nonidet P-40, 10 mM imidazole, 10% glycerol, with 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM benzamidine were applied to Ni²⁺-nitrotetrazolium-agarose (Qiagen). Bound protein was eluted from the column with lysis buffer containing 80 mM imidazole. CKIα tagged with the dual histidine six-histidine tag of pRSET-B (Invitrogen) was used in all assays except where specifically indicated. Untagged CKIα was purified on S-Sepharose (Amersham Pharmacia Biotech) as described (19). Histidine-tagged and untagged kinase were previously found to behave similarly in protein phosphorylation experiments (19).

Quantitative Immunoblot Analysis—Equal amounts of total protein from partially purified CKIα preparations were run on 10% SDS-PAGE and transferred to supported nitrocellulose (Amersham Pharmacia Biotech) as described previously (19). The membrane was blocked by incubation in TTBS (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween 20) containing 3% bovine serum albumin and then probed with affinity-purified polyclonal antibody UT31, added at a 1:1000 dilution in blocking solution as the primary antibody (20). The proteins of interest that reacted with UT31 were visualized with a secondary detection step of 125I-labeled protein A (Amersham Pharmacia Biotech) added at 10 nCi ml⁻¹ of blocking solution. A standard curve of serial dilutions of a single protein preparation was used to determine the relative concentration of all CKIα preparations tested. The results were visualized and quantitated by PhosphorImager (Molecular Dynamics).

Kinase and Phosphatase Assays—Kinase and phosphatase reactions were performed in buffer containing 100 or 250 µl ATP, 30 mM HEPES, pH 7.5, 7 mM MgCl₂, 0.5 mM dithiothreitol, and 2 µCi of [γ-32P]ATP in a final volume of 20 µl. The reaction mixtures were incubated for 5 min at 37 °C, and then the reactions were stopped by the addition of SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography as described previously (1, 24). All assays were performed at least twice with good interassay reproducibility.

All phosphatase reactions were performed for 15 min at 37 °C in 30

| Mutation name | Primer sequence | Restriction site |
|---------------|-----------------|-----------------|
| D396          | 5'-TGGGATCCGGAGACTTTATTGCCCGAGATGGTC-3' | BsaI (-) |
| D393          | 5'-GCACTGTCATCTCCCTGAGGAGATGGTC-3' | NheI (+) |
| D370          | 5'-CAGCTTCTCTCTCTCTCTGAGGAGATGGTC-3' | SallI (-) |
| D380          | 5'-CTGGCTGAGAGGAGATGGTC-3' | NheI (+) |
| D349          | 5'-GGAGGAGGAGATGGTC-3' | Accl (+) |
| D329          | 5'-CTGCTGAGGAGATGGTC-3' | AarI (+) |
| D319          | 5'-GGAGGAGATGGTC-3' | AarI (+) |
| D305          | 5'-CTGCTGTCATCTCCCTGAGGAGATGGTC-3' | SpeI (+) |
| M1            | 5'-GGAGGAGGAGATGGTC-3' | SacI (+) |
| M2            | 5'-GGAGGAGGAGATGGTC-3' | SacI (+) |
| M3            | 5'-CTGGCTGAGGAGATGGTC-3' | AarI (+) |
| M4            | 5'-CTGCTGTCATCTCCCTGAGGAGATGGTC-3' | DdeI (-) |
| M5            | 5'-CTGGCTGTCATCTCCCTGAGGAGATGGTC-3' | BglII (+) |
| M6            | 5'-GGAGGAGGAGATGGTC-3' | BsoI (-) |
| S368A         | 5'-CTGCTGTCATCTCCCTGAGGAGATGGTC-3' | PvuI (+) |
| S377A         | 5'-CTGCTGTCATCTCCCTGAGGAGATGGTC-3' | HphI (-) |
| 10xHis         | 5'-CTGGCTGTCATCTCCCTGAGGAGATGGTC-3' | None |

Table I

Primers used for site-directed mutagenesis

The primer sequences used for site-directed mutagenesis are listed in Table I. All primers are phosphorylated at their 5' ends and represent the anticoding strand of the CKIα gene.
FIG. 1. CKIe is autophosphorylated on similar sites in vivo and in vitro. A, plasmids encoding 6xHA-tagged CKIe, either wild type (WT, lanes b and c) or a kinase-inactive mutant (MUT, lanes d and f), under the control of the cytomegalovirus promoter were transiently transfected into HEK 293 cells. Empty vector (V, lanes a and d) was used as a control. At 2 days post-transfection, cultures were metabolically labeled with 2 μCi/ml H$_3$PO$_4$, in phosphate-free medium for 5 h. For the last 30 min of labeling, cultures were either not treated (lanes a–c) or treated (lanes d–f) with the cell-permeable phosphatase inhibitor calyculin A (50 nM). Cells were then lysed in radiolabeled CKIe, which was excised and rehydrated in 50 mM ammonium bicarbonate digestion buffer and spotted onto a cellulose plate. For maps performed in parallel, equal counts were spotted on each plate. Electrophoresis was resolved by SDS-PAGE on a 9% gel. Control experiments with 12CA5 monoclonal antibody and protein A-agarose and separated by SDS-PAGE on a 9% gel. Control experiments demonstrated equal expression of wild-type and mutant CKIe under these conditions (data not shown). Radioactive proteins were visualized by PhosphorImager analysis. The sites of CKIe predicted migration is indicated by an open circle. CKIe with altered mobility is indicated by a filled circle. B, immunoprecipitated in vivo 32P-labeled CKIe was excised from the lanes shown in A and analyzed by two-dimensional phosphopeptide mapping. Phosphopeptides were visualized by PhosphorImager. Recombinant CKIe autophosphorylated in vitro is shown in panel g. Specific phosphopeptides are indicated with letters (generally above the spot) for clarity.

mm HEPES, pH 7.5, 7 mM MgCl$_2$, and 200 μg of ml$^{-1}$ bovine serum albumin and contained 8–12 ng of the catalytic subunit of PP2A unless otherwise noted. Protein concentration was determined by the method of Bradford (25).

In Vitro Autophosphorylation and Two-dimensional Peptide Mapping—Partially purified CKIe proteins were radiolabeled in vitro or, for Fig. 1, immunoprecipitated from 32P-labeled cells. Approximately 20–50 μg of each kinase were treated with 1 μg of PP2A, for 15 min at 37 °C. Phosphatase activity was blocked by the addition of 200 nM okadaic acid, and the kinase was allowed to re-autophosphorylate in the presence of [γ-$^{32}$P]ATP for 15 min at 37 °C. The kinase reactions were resolved by SDS-PAGE on a 10% gel. Protein was stained briefly with Coomassie Brilliant Blue, the gels were dried, and the labeled proteins was visualized by autoradiography. Radiolabeled protein bands were excised and rehydrated in 50 mM ammonium bicarbonate digestion buffer. The gel slices were microwaved, 10 μg of trypsin was added, and digestion was carried out for 20 h at 37 °C. The buffer was removed from the gel slices and Cerenkov-counted to determine recovery of trypsin phosphopeptides. The digest was then lyophilized to dryness.

The two-dimensional peptide mapping method of Van Der Geer et al. (26) was used to separate phosphopeptides of CKIe. Plastic-backed 100-μm cellulose plates were obtained from Sigma. Lyophilized tryptic peptides of CKIe were suspended in 5–10 μl of pH 1.9 electrophoresis buffer and spotted onto a cellulose plate. For maps performed in parallel, equal counts were spotted on each plate. Electrophoresis was carried out at 1300 V for 30 min in pH 1.9 buffer containing 2.2% formic acid, and 7.5% acetic acid. Following electrophoresis, the cellulose plates were allowed to dry completely. Dried plates were subjected to ascending chromatography for 3 h in phosphochromatography buffer containing 37.5% n-butanol, 25% pyridine, and 7.5% acetic acid. Phosphopeptides were visualized by PhosphorImager (Molecular Dynamics).

FIG. 2. Site-directed mutants of CKIe. CKIe carboxyl-terminal tail residues 298–414 showing the locations of introduced truncation and point mutations. The locations of stop codons generated by site-directed mutagenesis are indicated by ball-and-stick symbols. Point mutations of serine and threonine residues are indicated by the names of the primers used for mutagenesis (Table I). Predicted tryptic cleavage sites are denoted by filled triangles.

RESULTS

CKIe in Vivo Phosphorylation—CKIe activity is regulated in vitro by carboxyl-terminal tail autophosphorylation; in vivo the autophosphorylated kinase is rapidly dephosphorylated in a futile cycle of autophosphorylation and dephosphorylation (13). To determine whether CKIe was autophosphorylated in vitro and in vivo on the same sites, two-dimensional phosphopeptide maps were prepared from CKIe autophosphorylated in vitro (Fig. 1A and B, panels a–f) and in vivo (Fig. 1B, panel g). In vivo autophosphorylated CKIe was immunoprecipitated from transiently transfected human embryonic kidney 293 cells metabolically labeled with H$_3$[$^{32}$P]PO$_4$. The rapid turnover of phos-
phate on CKI\(\varepsilon\) in vivo autophosphorylation sites (13) was blocked by addition of the cell-permeable phosphatase inhibitor calyculin A to selected cells for the last 30 min of labeling. As Fig. 1A, lanes c and f, shows, immunoprecipitated kinase-inactive CKI\(\varepsilon\) (K38R) appears minimally phosphorylated in vivo. Phosphopeptide mapping demonstrates that the kinase-inactive CKI\(\varepsilon\) is phosphorylated predominantly on a single peptide (spot f in Fig. 1B) and that phosphorylation is not altered substantially by the addition of the phosphatase inhibitor calyculin A (Fig. 1B, panels c and f).

In vivo, wild-type CKI\(\varepsilon\) is minimally phosphorylated, and on the same peptide f in the absence of calyculin A, with low levels of autophosphorylation on additional peptides i and h (compare Fig. 1B, panels b and c). The addition of calyculin A to transfected cells inhibits a number of endogenous serine/threonine phosphatases and leads to a marked increase in autophosphorylation of CKI\(\varepsilon\) on additional sites (Fig. 1A, lanes b and e, and peptides d, e, g, and m in Fig. 1B, panel e). The phosphorylation of CKI\(\varepsilon\) in vivo in the presence of calyculin A is primarily autophosphorylation, because the phosphorylation of kinase-inactive CKI\(\varepsilon\) is not increased significantly by the phosphatase inhibitor (compare lanes c and f, Fig. 1A). Previous studies have established that the autophosphorylation of CKI\(\varepsilon\) in vitro and in vivo is intramolecular (13). CKI\(\varepsilon\) appears to autophosphorylate on the same peptides in vitro as it does in vivo, as the phosphopeptide maps of the kinase labeled either way are very similar (compare panels e and g, Fig. 1B). In addition, peptide maps prepared from bacterially expressed protein (Fig. 1B, panel g) demonstrate two additional phosphopeptides, labeled j and k. In vitro labeled protein may contain these extra phosphopeptides because of more extensive autophosphorylation in vitro, or the sites may be phosphorylated in vivo but not detected because they are rapidly dephosphorylated by a cellular phosphatase that is not inhibited by calyculin A. Phosphopeptides a, b, and c appear to be nonspecific, as they appear in the absence of kinase as well (compare Fig. 1B, panel d with panels e and f). Because the CKI\(\varepsilon\) in vivo and in vitro autophosphorylation sites appear to be similar, bacterially expressed in vitro autophosphorylated protein was used for phosphopeptide mapping experiments.

Truncation Mutagenesis of the CKI\(\varepsilon\) Carboxyl-terminal Tail—Autophosphorylated CKI\(\varepsilon\) can be activated up to 20-fold by treatment with active PP2A. Previous truncation and domain-swap experiments have indicated that autophosphorylation...
tion sites in the carboxyl-terminal tail of CKIε and CKIδ are responsible for this autophosphorylation-dependent autoinhibition (13, 19, 20). To determine the specific regions of CKIε required for phosphorylation-dependent autoinhibition, a series of histidine-tagged carboxyl-terminal truncation mutants of CKIε were generated by site-directed mutagenesis (Fig. 2 and Table II). These truncated active kinases were expressed in E. coli and partially purified by metal-chelate chromatography, and CKIε protein levels were normalized by quantitative immunoblot (Fig. 3A). The kinases as purified from E. coli were substantially autophosphorylated and hence autoinhibited (19).

To determine which regions within the CKIε carboxyl-terminal tail were important for phosphorylation-dependent inhibition, the activity of the recombinant truncated autoinhibited kinases on SV40 large T antigen was determined before and after their activation by PP2A. As shown in Fig. 3B, the activity of full-length CKIε and truncation mutants D383, D370, and D360 was stimulated up to 15-fold toward T antigen by pre-treatment with PP2A, whereas truncation mutants D349, D329, D319, and D305 were activated only 3-fold. These results were reproducible using T antigen as a substrate, and similar results were obtained when the CKIε truncation mutants were used to phosphorylate casein (data not shown).

Removal of the inhibitory carboxyl-terminal domain of CKIε by limited trypsin proteolysis has previously been shown to increase the specific activity of CKIε 3-fold (19). To determine whether recombinant truncated forms of CKIε would show a similar increase in activity relative to full-length CKIε, full-length CKIε and truncation mutants D383, D370, D360, D349, D329, and D305 were tested for their ability to phosphorylate IκBα (19). The truncation mutant D349 was approximately 2.5-fold more active than truncation mutant D360 (Fig. 4). These results suggest that a phosphorylation-dependent inhibitor of CKIε activity lies between or near residues 349–360 of the CKIε carboxyl-terminal tail.

The simplest explanation for these results is that inhibitory autophosphorylation sites are located between residues 349 and 360. To test this, the putative phosphoacceptor residues (Ser-350, Thr-351, and Ser-354) in this region were mutated (in the background of full-length CKIε) to alanine (mutant M3, Fig. 2 and Table II), and the resulting mutant kinase was expressed and tested. However, mutant M3 showed no significant decrease in the ability to be activated by phosphatase (data not shown) nor was there any detectable increase in the specific activity of the enzyme (Fig. 5). Additionally, two-dimensional phosphopeptide mapping of the M3 protein showed no change in phosphopeptides (data not shown). One potential explanation of the data is that this region may inhibit kinase activity or kinase-substrate interaction by interaction with inhibitory phosphoryl groups more proximal to the kinase domain.

Two-dimensional Peptide Maps of CKIε—To identify the specific sites of CKIε autophosphorylation, the panel of truncation mutants was further analyzed. Mutant proteins expressed in E. coli and purified as described were first treated with PP2A to remove phosphoryl groups placed by autophosphorylation during expression and purification. Phosphatase activity was then inhibited by the addition of okadaic acid, and the kinase was allowed to autophosphorylate in the presence of 250 μM γ-32PATP. Autophosphorylated kinases were isolated by SDS-PAGE and phosphopeptide-mapped as described under “Materials and Methods.” This procedure allowed the preferential radiolabeling of phosphoacceptor sites sensitive to PP2A and hence sites implicated in autoinhibition and relief of autoinhibition by phosphatase treatment (19). Truncations that lead to loss of phosphopeptides were further analyzed by introduction of mutations in the implicated region, converting specific serine and threonine residues to alanine. All informative phosphopeptide maps were performed at least twice with similar results.

Fig. 6, A and B, illustrates the identification of potential autophosphorylation sites. Truncation mutant D319 lacks a single phosphopeptide (indicated by an arrow) present in truncation mutant D329 and full-length CKIε, suggesting there are phosphorylation sites in that interval (Fig. 6). The two potential phosphorylatable residues, Ser-323 and Thr-325, were therefore mutated to alanine in full-length CKIε, and the resulting protein (designated M1) was expressed, autophosphorylated, and phosphopeptide-mapped. As the arrow indicates, mutant M1 lacks a phosphopeptide present in the wild-type.
FIG. 6. Mutants S323A/T325A and S368A/S377A eliminate autophosphorylation sites. Phosphopeptide maps of CKIα mutant proteins. The indicated truncation or alanine substitution mutants were treated with PP2A, and then allowed to autophosphorylate in the presence of [γ-32P]ATP prior to peptide mapping. In each panel, truncation mutants are compared with full-length (FL) CKIα and a full-length point mutant where potential phosphoacceptor residues were mutated to alanine. Arrows indicate phosphopeptides that disappear upon truncation or point mutagenesis. The pertinent region of the CKIα primary sequence is displayed in single-letter amino acid code. Triangles denote predicted trypsin cleavage sites. Stop codons inserted by mutagenesis are indicated by ball-and-stick symbols labeled with the number of the residue that becomes the last amino acid of the polypeptide. A, analysis of truncations between residues 329 and 319. M1 is the full-length CKIα with S323A and T325A. B, analysis of truncations between residues 360 and 383. A solid arrow indicates peptide absent in D383; an open arrowhead indicates peptide of aberrant migration in D370 as described under “Results.”

protein, strongly suggesting that Ser-323 and/or Thr-325 are autophosphorylation sites.

Phosphopeptide mapping results for truncations between residues 360 and 383 (Fig. 6B) permit the assignment of specific autophosphorylation site, Ser-368. Both D360 and the double mutant S368/377A lacked a specific phosphopeptide (Fig. 6B, solid arrow) present in full-length and D383 CKIα. A phosphopeptide not present in D360 is apparent in the D370 mutant, albeit at a faster vertical mobility than in full-length or D383 CKIα (open arrow). Closer examination of the primary amino acid sequence in this region revealed that the D370 stop codon was introduced one amino acid carboxyl-terminal to a potential trypsin cleavage site. Trypsin cleaves inefficiently very close to the ends of polypeptides (27); therefore, the aberrant migration observed for D370 is probably because the trypsin site following residue 369 was not used in the D370 mutant, resulting in a peptide longer by several residues, including a very close to the ends of polypeptides (27); therefore, the aberrant migration observed for D370 is probably because the trypsin site following residue 369 was not used in the D370 mutant, resulting in a peptide longer by several residues, including a valine residue that increased the hydrophobicity of the peptide. To confirm this hypothesis, digestion of radiolabeled D370 was repeated. A fraction of the phosphopeptide with aberrant migration could be shifted to the position predicted by D383 and full-length CKIα upon digestion with a 10-fold higher trypsin concentration than previously used. These data are consistent with Ser-368 being an autophosphorylation site.

Autophosphorylation sites in the carboxyl-terminal tail of CKIα have been localized to residues Ser-323 and/or Thr-325 (M1, Fig. 6A), Ser-368 (Fig. 6B), Thr-334, and/or Thr-337 (M2), and Ser-405, Thr-407, and/or Ser-408 (M5) (data not shown). In most cases, the site of autophosphorylation was identified as...
one of 2–3 residues mutated in a multiple point mutant (Table II).

Effect of CKIe Phosphoacceptor Site Mutagenesis—Phosphopeptide mapping data were used to choose mutation sites for generating a nonphosphorylatable tail mutant of CKIe. Primers M1, M2, M5, and S368A were used for simultaneous site-directed mutagenesis of CKIe. The resulting multiple mutant, MM2 (S323A/T325A/T334A/T337A/S368A/S405A/T407A), was sequenced to confirm the presence of planned mutations and the absence of adventitious changes, and it was then expressed, partially purified, and autolabeled. A two-dimensional peptide map of MM2 indicates that these mutations lead to a CKIe molecule with markedly reduced autophosphorylation (Fig. 7A).

To determine whether a lack of tail autophosphorylation correlates with an increase in catalytic activity, wild-type CKIe and the MM2 mutant were normalized by their kinase activities against IxBo (Fig. 7B). However, when the amount of kinase in each reaction was checked by quantitative immunoblot, a dramatic difference was seen between the amount of mutant MM2 CKIe and wild-type CKIe. Kinase activity on IxBo was normalized to the amount of kinase detected by immunoblot (Fig. 7C) and graphed (Fig. 7D). About 8-fold less MM2 kinase is required to achieve the same amount of IxBo phosphorylation as wild-type CKIe. This activation is more pronounced than was seen by complete elimination of the carboxyl-terminal domain (Fig. 4).

DISCUSSION

CKIe belongs to a family of ubiquitous protein kinases with an emerging role in regulation of transcription, DNA replication and DNA repair. A mechanism for the potential regulation of CKIe and related CKIα has been autophosphorylation. In the current study we have mapped the autophosphorylation sites of CKIe in the carboxyl-terminal inhibitory domain and demonstrated that a multiple phosphorylation site mutant has an 8-fold increase in kinase activity on the substrate IxBo. Additionally, a region between amino acids 349 and 360 was identified as a negative regulator of kinase activity. This constitutively active mutant now allows us to test the role of inhibitory autophosphorylation in the potential biologic functions of CKIe including processes such as circadian rhythm and DNA replication.

It was noted previously that truncation and dephosphorylation of CKIe do not produce the same degree of activation (19). However, this discrepancy was attributed to incomplete truncation of the inhibitory domain or the presence of an inhibitory phosphorylated residue in the kinase domain. The latter possibility has not been ruled out, as the CKIe D219 truncation mutant still autophosphorylates (Fig. 6A) and the D305 truncation mutant is still activated two-fold by phosphatase treatment (19). Further supporting the presence of an inhibitory phosphorylation site in the kinase domain, Kuret and co-workers (28) described two forms of recombinant yeast Cki1 kinase domain; one form was autophosphorylated in the kinase domain and had a 4-fold decrease in activity compared with unphosphorylated Cki1. Thus, inhibition of kinase activity via phosphorylation of the kinase domain may be a common feature of the CKI family. It is notable that CKI is one of the few serine/threonine kinases that do not require phosphorylation on their T-loop for full kinase activity (29). The data therefore suggest that there are inhibitory autophosphorylation sites within the kinase domain of several CKI family members.

Using recombinant mutants of CKIe, including carboxyl-terminal truncations and point mutations of putative phosphoacceptor residues in the carboxyl-terminal tail region, a two-dimensional peptide mapping approach was used to identify sites of autophosphorylation (Fig. 8). In previous peptide phosphorylation experiments, the preference of CKI for an acidic residue or phosphate group three residues amino-terminal to the phosphoacceptor was well characterized (14, 15, 30–32). Interestingly, the sites mapped in this study do not match consensus CKI sites. Autophosphorylation sites were found scattered throughout the CKIe carboxyl-terminal tail. In two of the mutants altering CKIe autophosphorylation sites, M2 (T334A,T337A) and M5 (S405A,T407A,S408A), at least two phosphoacceptor residues are oriented 3 residues apart. These sites are in regions of high homology to CKIα. It is possible that phosphorylation on the amino-terminal residue of the series could be a catalyst for phosphorylation of the phosphoacceptor carboxyl-terminal to the first residue. However, if this were the case, the first phosphorylation event would still have taken place without any upstream acidic region to direct it. It may be that the high local concentration of the tail is more important in determining specific phosphorylation sites. Alternatively, it may be that three-dimensional structure of the tail is more important than upstream acidic character. In support of this model, CKI is able to phosphorylate specific residues in the amino terminus of SV40 large T antigen only in the context of full-length protein. The same T antigen residues were not phosphorylated by CKI when present in peptides or in T antigen domains (24).

It appears that mammalian cells go to considerable lengths to ensure that CKIe remains in a dephosphorylated, active form, suggesting that its activity is either required or modulated by some other means. Thus far, no agents except the phosphatase inhibitors okadaic acid and calyculin A have been identified as instigating CKIe autophosphorylation in vivo. The link between the CKIe and DNA damage-responsive pathways in yeast (3), and recently in mammals (17), and the link between CKIe and circadian rhythm in Drosophila suggest that a DNA damage or circadian rhythm-regulated event triggers up- or down-regulation of CKI activity. The identification of CKIe autophosphorylation sites described above may provide a means to determine the role of CKIe autophosphorylation on the in vivo regulation of this enzyme.

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