Role of COOH-terminal Phosphorylation in the Regulation of Casein Kinase Iα*

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Casein kinase Iα is a member of the casein kinase I (CKI) family, a group of second messenger independent protein kinases. We present evidence that the COOH-terminal domain of CKIα has regulatory properties. CKIα expressed in Escherichia coli was activated by heparin, as found previously, and by treatment with the catalytic subunit of type-1 protein phosphatase (CS1). Concomitant with activation by CS1, there was a reduction in the apparent molecular weight of CKIα from 55,000 to 49,000 as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Truncation of CKIα by removal of the COOH-terminal 110 amino acids eliminated the ability of CS1 to activate or to increase electrophoretic mobility. Casein kinase Iα, a 37-kDa isozyme that lacks an extended COOH-terminal domain, was not activated by CS1 or the presence of heparin. However, a chimeric enzyme consisting of CKIα fused to the COOH-terminal domain of CKIα was activated by both heparin and CS1. Analysis of the effects of CS1 on a series of CKIα COOH-terminal truncation mutants identified an inhibitory region between His317 and Pro322, which contained six potential phosphorylation sites. From analysis of the specific activities of these truncation mutants, removal of the same region resulted in enzyme with a specific activity nearly 10-fold greater than wild-type. Thus, CKIα activity can be regulated by phosphorylation of its COOH terminus, which may serve to create an autoinhibitory domain. This mechanism of regulation could have important consequences in vivo.

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1 The abbreviations used are: CKI, casein kinase I; CS1, catalytic subunit of type-1 protein phosphatase; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); TLCK, N-α-tosyl-L-lysine chloromethyl ketone.

‡ L. Robinson, personal communication.
substrates in which the phosphate group is found in the sequence motif (Ser(P)/Thr(P))-Xaa-Xaa-(Ser/Thr) (26, 28). CKI's ability to utilize a phosphate group as a recognition determinant could link its activity to that of other protein kinases, which in turn could be regulated by classical second messengers (29). Some effective CKI substrates do not require prior phosphorylation. The clearest example here is inhibitor-2 of protein phosphatase 1 (30) which contains a cluster of acidic residues NH2-terminal to the target site.

Relatively little is known about the control of CKI enzymes. In this report, we define an autoinhibitory region in the COOH terminus of CKIα through which activity can be modulated by phosphorylation.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutations were introduced into the CKIα cDNA (10) using the Sculptor in vitro mutagenesis system (Amersham Corp.) according to the manufacturer's instructions. Three COOH-terminal truncation mutants were made by insertion of artificial stop codons at positions 328, 343, and 364 and designated CKIα(L327), CKIα(L342), and CKIα(L363), respectively. The following oligonucleotides were used for the mutagenesis with mutated bases underlined: CKIα(L327), 5′-GGCTTCCTCTTAAACAGTTCG; CKIα(L342), 5′-AGTGGTCTCTTCAATGGTC; and CKIα(L363), 5′-GAGAAGCCCGGTCA. To form an α-δ chimeric kinase, a Dral site (underlined) was created at the stop codon (1355 bp) of CKIα DNA (7) using the oligonucleotide 5′-CCACAGGTTTTAAAGCATGAAT. All mutations were confirmed by nucleotide sequence analysis according to the method of Sanger (31).

Expression Vector Construction—All COOH-terminal truncation mutants of CKIα were subcloned into the pET-8c (32) expression vector as described previously (10). To create the α-δ chimera, mutant CKIα DNA was digested with NdeI and Dral and Dral to produce a 974-bp coding region fragment. This fragment encoded the entire amino acid sequence of CKIα (amino acids 1–325). To create the δ portion of the chimera, the polymerase chain reaction (GeneAmp, Invitrogen) was used to amplify a product of 365 bp from 1251 to 1616 bp of the CKIα cDNA (10), according to the manufacturer's instructions. The oligonucleotides 5′-CGCTTCCGAATATCCACCTGCTGGC and 5′-CGCTCAGATCCGGTGGATC were used for the polymerase chain reaction with the underlined sequence indicating the Nrlu and BamHI sites of the sense and antisense primers, respectively. This polymerase chain reaction product, which encoded a portion of the COOH-terminal region of CKIα (amino acids 319–428), was digested with Nrlu and BamHI and ligated to the 974-bp CKIα fragment producing a fragment of 1339 bp. The 1339-bp fragment was then ligated to the pET-3c expression vector previously cut with NdeI and BamHI. The entire polymerase chain reaction product and all junctions formed were confirmed by nucleotide sequence analysis (31).

Expression and Purification of CKIα—The CKIα COOH-terminal truncation mutants and the α-δ chimera were expressed, harvested, and lyed as described previously for wild-type CKIα (10). Wild-type CKIα, the α-δ chimera, and the COOH-terminal truncation mutants were purified using a combination of S Sepharose (Pharmacia Biotech Inc.) and γ-ATP-agarose chromatography, an affinity resin for the purification of protein kinases (33). Supernatant from Escherichia coli cell lysate (25–50 ml) was batch-absorbed for 2 h at 4 °C with 2–3 ml of S Sepharose previously equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM TLCK, and 1 mM dithiothreitol). The resin was collected by centrifugation at 5000 × g for 10 min, transferred into 5 ml of buffer B (Whatman), and washed with 5 column volumes of buffer A. Enzyme was step-eluted using 0.6 M NaCl in buffer A, and fractions containing CKIα activity were pooled and diluted to <0.1 mM NaCl. γ-ATP-agarose was equilibrated with buffer B (50 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 20 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM TLCK, and 1 mM dithiothreitol) and then washed by 5 column volumes of buffer B in vitro with most of the protein kinase activity being removed. 32P-Labeled species were excised and subjected to Cerenkov scintillation counting.

Protein Phosphatase Reactions—Phosphatase reactions were performed using conditions as described for kinase assays except for the addition of 50 μM [γ-32P]ATP (1000–5000 cpm/pmol). For rephosphorylation of CKIα following CS1 treatment, CKIα was incubated at 30 °C for 1 h in the kinase assay buffer with ATP and 32P-Labeled protein was digested for 12 h with endolysine C at a ratio of 1:20 in 75 mM Tris-HCl, pH 8.0. The mixture was subjected to 20% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore), and detected by staining with Coomassie Blue and autoradiography. Radioactive protein bands were excised, and the proteins were hydrolyzed for 90 h in 6 N HCl. Phosphoamino acids were resolved by thin layer electrophoresis for 67 min at 500 V in 5% acetic acid, 0.5% pyridine, pH 3.5, and detected by 0.5% ninhydrin and autoradiography as described previously (39). To localize autophosphorylation sites, CKIα was allowed to autophosphorylate in the presence of 50 μM [γ-32P]ATP (1000–5000 cpm/pmol) as described above. 32P-Labeled protein was subjected to 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and detected by staining with Coomassie Blue and autoradiography. Radiolabeled protein bands were excised, and the proteins were hydrolyzed for 90 h in 6 N HCl. Phosphoamino acids were resolved by thin layer electrophoresis for 67 min at 500 V in 5% acetic acid, 0.5% pyridine, pH 3.5, and detected by 0.5% ninhydrin and autoradiography as described previously (39). To locate autophosphorylation sites, CKIα was allowed to autophosphorylate in the presence of 50 μM [γ-32P]ATP (1000–5000 cpm/pmol), and 32P-Labeled protein was digested for ~12 h with endolysine C at a ratio of 1:20 in 75 mM Tris-HCl, pH 8.0. The mixture was subjected to 20% SDS-PAGE, transferred to polyvinylidene difluoride membrane and autoradiography performed. 32P-Labeled species were excised and subjected to the Edman degradation reaction for NH2-terminal protein sequencing (40).

Miscellaneous Methods—For phosphoamino acid analysis, CKIα was allowed to autophosphorylate in the presence of 50 μM [γ-32P]ATP (1000–5000 cpm/pmol) as described above. 32P-Labeled protein was subjected to 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and detected by staining with Coomassie Blue and autoradiography. Radiolabeled protein bands were excised, and the proteins were hydrolyzed for 90 h in 6 N HCl. Phosphoamino acids were resolved by thin layer electrophoresis for 67 min at 500 V in 5% acetic acid, 0.5% pyridine, pH 3.5, and detected by 0.5% ninhydrin and autoradiography as described previously (39). To localize autophosphorylation sites, CKIα was allowed to autophosphorylate in the presence of 50 μM [γ-32P]ATP (1000–5000 cpm/pmol), and 32P-Labeled protein was digested for ~12 h with endolysine C at a ratio of 1:20 in 75 mM Tris-HCl, pH 8.0. The mixture was subjected to 20% SDS-PAGE, transferred to polyvinylidene difluoride membrane and autoradiography performed. 32P-Labeled species were excised and subjected to the Edman degradation reaction for NH2-terminal protein sequencing (40).
RESULTS

Autophosphorylation of Casein Kinase 1α—CK1α can be expressed as a soluble active enzyme in E. coli (10). However, CK1α expressed in E. coli had an apparent molecular weight (M̄) of −55,000 on SDS-PAGE, significantly larger than the M̄ of 49,121 predicted from its amino acid sequence (Fig. 1, lane 1). Since phosphorylations of proteins is known to retard electrophoretic mobility, we treated CK1α with CS1. The apparent M̄ of CK1α was reduced from −55,000 to −49,000 (Fig. 1, lane 2). Moreover, if CK1α was subsequently incubated with ATP, Mg²⁺, and microcystin to inhibit the phosphatase, its electrophoretic mobility decreased yielding a polypeptide apparent M̄ of 49,000 (Fig. 1, lane 3). Phosphoamino acid analysis of this autophosphorylated CK1α revealed phosphoserine and phosphothreonine but no phosphotyrosine (data not shown). Furthermore, expression of a "kinase-dead" mutant of CK1α (K₃⁸⁸N) resulted in inactive protein with apparent M̄ of −49,000, suggesting that phosphorylations affecting mobility were due to autophosphorylation within the E. coli cells. We conclude that recombinant CK1α is a phosphoprotein and that its phosphorylation state affects its migration on SDS-PAGE. At earlier stages of the CK1α phosphorylation reaction, several intermediate species could be identified with M̄ values in the range of 49,000–55,000, suggesting phosphorylation at multiple sites (data not shown). To determine whether the phosphorylation state of CK1α affected its activity toward exogenous substrates, recombinant CK1α was treated with CS1 and protein kinase activity was measured as described under "Experimental Procedures." Incubation with CS1 led to progressive activation of the enzyme with maximal activation of 2–3-fold after 1 h (Fig. 2). This activation correlated with dephosphorylation of CK1α antibody as described under "Experimental Procedures." An autoradiogram is shown. Lane 1, untreated recombinant CK1α; lane 2, CK1α after treatment with CS1; lane 3, sample from lane 2 incubated with ATP, Mg²⁺, and 1 μM microcystin.

Preceding 32P-labeled subject was subjected to protein sequence. A unique CK1α sequence, FGA, was obtained, which corresponds to amino acids Phe²⁹⁵–Ala²⁹⁷. The next lysine residue occurs at Lys³⁶⁸, thus localizing these in vitro autophosphorylation sites to the region from residue 295 to 368. In other experiments, the initial rate of CK1α autophosphorylation was shown to be first order with respect to protein concentration over a 20-fold range of enzyme (2.5–50 μg/ml) (data not shown). Thus, the specific activity is independent of concentration, suggesting that in vitro autophosphorylation of CK1α occurs via an intramolecular mechanism.

Regulatory Role of the COOH Terminus of CK1α—Previously, we reported that CK1α activity toward certain substrates, such as the D4 peptide, could be increased 4–5-fold in the presence of heparin, whereas a truncated form of the enzyme (CK1αΔ317), lacking the COOH-terminal 111 amino acids, was unaffected (10). Similarly, D4 peptide phosphorylation by CK1α, an isoform with a minimal COOH-terminal extension, was not activated by heparin and in fact was inhibited by −50% (7). Therefore, we constructed an α-δ chimeric enzyme, CK1αδ, consisting of the entire 325 amino acids of CK1α fused to the COOH-terminal 110 amino acids of CK1δ. The chimeric enzyme had a calculated M̄ of 49,609, but, like CK1α, it had an apparent M̄ of −55,000 kDa on SDS-PAGE, suggesting that it had undergone autophosphorylation in E. coli (data not shown). Using the D4 peptide as substrate, CK1α activity was measured in the presence of heparin or after treatment with CS1 (Fig. 3). CK1α was inhibited by heparin and showed only 39% activation by CS1. CK1αΔ317 was not activated by heparin or CS1. CK1αδ, in contrast, was activated 3–4-fold by both heparin and CS1 as was CK1αδ. This result indicates that the COOH-terminal region of CK1α is necessary and sufficient to confer heparin and CS1 activation to the enzyme.

Since there was a correlation between heparin and CS1 activation of CK1α (Fig. 3), we determined whether the phosphorylation state of CK1α affected its activation by heparin. CK1α was treated with CS1, and then protein kinase activity was measured with increasing concentrations of heparin. Once CK1α was activated by CS1, it could no longer be activated by heparin (Fig. 4). This finding suggests that heparin and CS1 activate CK1α, at least in part, via a common mechanism.

Localization of Autophosphorylation Sites and an Autoinhibitory Domain in CK1α—To localize the sites already phosphorylated in CK1α purified from E. coli, three COOH-terminal truncations were created by site-directed mutagenesis (Fig. 5). In addition, we already had the CK1αΔ317 construct (10). These mutants, designated CK1αΔ317, CK1αΔ327, CK1αΔ342, and

³ P. R. Graves and P. J. Roach, unpublished results.
Thus, sites contributing to the anomalous migration of CKI

d
CKI terminus. The effects of CS1 on activity were also monitored.

on polyacrylamide gels are distributed throughout the COOH

terminus. The effect of 100 μg/ml heparin (cross-
hatched bars) or incubation with CS1 for 60 min (solid bars) on D4

treated, a greater degree of activation was observed, 2-fold in

CS1 (Fig. 7). Each mutant protein was treated with CS1 and

subjected to SDS-PAGE. While CKI

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autophosphorylation sites are

increased mobility (Fig. 5), which had not been exposed to phosphatase.

Amino acids His

317 to Met

364 of CKI

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are shown. Potential autophosphorylation sites are underlined; arrows denote the locations of the different truncations described in this study.

CKIΔ363, were individually expressed and purified from E.
coli (Fig. 6). Each mutant protein was treated with CS1 and

subjected to SDS-PAGE. While CKIΔ317 showed no change in
electrophoretic mobility after CS1 treatment, CKIΔ327, CKIΔ342, and CKIΔ363 all showed increased mobility (Fig. 6). Thus, sites contributing to the anomalous migration of CKI

don polyacrylamide gels are distributed throughout the COOH

terminus. The effects of CS1 on activity were also monitored.
CKIΔ317, as shown previously in Fig. 3, was not activated by

CS1 (Fig. 7A). As the COOH terminus was progressively extended, a greater degree of activation was observed, 2-fold in

CKIΔ327 and 4-fold in CKIΔ342. However, the addition of further COOH-terminal sequences, in CKIΔ363 and full-

length CKI

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, correlated with no greater activation (Fig. 7A).

These results suggest that there are minimally two inhibitory autophosphorylation sites, one between residues His

317 and Pro

342, and another between residues Pro

327 and Pro

342. There are six candidate sites in these regions, Ser

318, Thr

323, Ser

328, Thr

329, Ser

331, and Thr

337 (Fig. 5). Also, not all of the sites that influence electrophoretic mobility affect activity.

COOH-terminal deletion itself affected the activity of CKI

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as judged by the specific activities of the purified truncated proteins (Fig. 7B). Thus, CKIΔ317, in which essentially all of the COOH-terminal tail was removed, had a specific activity nearly 10-fold greater than that of wild-type enzyme. CKIΔ327 had a specific activity 5-fold greater than wild-type. However, truncations up to residue 342 did not alter the specific activity compared with wild-type enzyme. Thus, CKI

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contains an autoinhibitory region between His

317 and Pro

342 (Fig. 5), the same region identified as inhibitory from CS1 treatment (Fig. 7A).

DISCUSSION

Casein kinase I has, for many years, been considered to be a

constitutively active enzyme since it is spontaneously active

after isolation from native tissues or after expression of recombi-

nant enzyme in a prokaryotic system. Moreover, this sponta-

eaneous activity is not generally found to be affected by second

messengers or by association with any known proteins. There

is a report of the inhibition of a 37-kDa CKI species by phos-

phatidylinositol bisphosphate (41), although this result has

been questioned by others (42). In the present study, we pro-

vide evidence that the CKI

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isoform is a regulatable enzyme whose COOH terminus acts as an autoinhibitory domain in which phosphorylation at Ser and Thr residues causes inacti-

vation of the enzyme.

The CKI family consists of isoforms with a conserved cata-

talytic domain and variably sized NH2- and COOH-terminal ex-

tensions. The NH2-terminal extensions are less than 15 amino

acids, except in the case of the recently identified γ isoforms (9),

with lengths of ~43 amino acids, and the yeast Yck1p and

Yck2p enzymes (12, 13), with ~73-amino acid extensions. The

COOH-terminal domains of most CKI isoforms are consid-

erably larger. For example, two of the mammalian (9, 10) and six of the yeast (11–16) CKI isoforms have COOH-terminal tails of at least 100 amino acids. CKIα and β, which are most likely the commonly studied 37-kDa forms of CKI, have COOH-terminal domains of only 13–25 amino acids (6).

The first indication that the COOH-terminal region of CKI

might be regulatory came from earlier studies with heparin.

Effects of heparin on CKI isoforms are complicated and sub-

strate-specific (see Ref. 10). However, we observed that full-

length CKI

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was activated by heparin when the D4 peptide was

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creating a chimeric kinase consisting of CKI conferred the heparin activation and tested the hypothesis by used as a substrate, whereas mutant CKI isoform, with its minimal COOH-terminal extension, was not activated by heparin and in fact was partially inhibited (Fig. 3; Ref. 7). The activation is normalized to the activity measured without dephosphorylation. B, the specific activity of the untreated samples using the D4 peptide as substrate.

That CKI, like most protein kinases, can autophosphorylate has been known for many years but had never been linked with any change in enzyme activity (43, 44). We previously reported that recombinant CKIα purified from E. coli could autophosphorylate without change in electrophoretic mobility (10). It is now apparent that CKIα does contain autophosphorylation sites that affect both electrophoretic mobility and, more importantly, activity toward exogenous substrates. The key observation was that treatment of purified recombinant CKIα with type 1 protein phosphatase activated the enzyme and reduced its apparent Mr, from 55,000 to its predicted value of 49,000. Thus, the sites that influence both activity and mobility are already modified, the phosphorylation presumably occurring inside the E. coli cells. This phosphorylation could be due to autophosphorylation or the action of other protein kinases. Arguing against the latter possibility is the observation that a kinase-dead mutant of CKIα (K39N) had apparent Mr, 49,000, indicative of the protein not being phosphorylated. Interestingly, a nuclear form of CKI (N1) was reported to have an apparent Mr, of 55,000 on SDS-PAGE (45) and not to autophosphorylate, which could be possible if the enzyme were already fully modified.

Using a series of COOH-terminal truncation mutants, we were able to localize the phosphorylation sites responsible for anomalous electrophoretic migration as being COOH-terminal to His317, and several different sites within this region contribute to this behavior. Similar results were observed with a completely different isoform, S. pombe CKI, in which anomalous migration on SDS-PAGE was attributed to the COOH-terminal domain (46). By analyzing the ability of protein phosphatase to activate the COOH-terminally truncated forms of CKIα, the inhibitory phosphorylation was localized to a region between His317 and Pro342, which contains six potential phosphorylation sites. Although we did not determine which of these six sites has the greatest inhibitory effect on the enzyme, multiple autophosphorylation sites are involved. Experiments with the same region, His317 to Pro342, was inferred to be autoinhibitory from consideration of the specific activities of the truncation mutants. Thus, activation of the enzyme by truncation may result from removal of inhibitory autophosphorylation sites. Heparin activates CKIα toward the D4 peptide by a mechanism that requires the COOH-terminal region. Possibly, heparin interacts with the COOH terminus, which carries a high positive charge, to cause an overall conformational change that mimics dephosphorylation. Heparin is unlikely to be a physiological regulator of the enzyme, but we cannot exclude the possibility that some other compound interacts with the regulatory COOH terminus. Likewise, it is not known in vivo whether the inhibitory phosphorylations discussed above would result from autophosphorylation or the action of a separate protein kinase. There is a parallel in the extracellular signal-regulated/mitogen-activated protein kinase enzymes, which can activate by autophosphorylation (47) even though enzymes of the MAPK or ERK family are thought to be responsible physiologically (48, 49).

Other CKI enzymes may also be regulated via their COOH-terminal domains, even though these have no sequence similarity whatsoever. For example, CKIγ3 can be activated 3-4-fold by type-1 protein phosphatase concomitant with a shift in its apparent molecular weight from 60 to 55 kDa on SDS-PAGE. Similarly, the S. pombe CKI isoform undergoes an inhibitory autophosphorylation that was localized to its COOH terminus (46). Truncation of the enzyme and removal of the COOH-terminal domain resulted in a 3-fold activation in the catalytic rate of the enzyme. Autophosphorylation, the majority of which was localized to the COOH-terminal domain, resulted in a 4-fold decrease in the affinity for protein substrate (46). Inhibitory COOH-terminal phosphorylation could therefore be

4 L. Zhai, P. R. Graves, and P. J. Roach, unpublished results.
a common regulatory mechanism for CKI isomers.

Given the unique substrate recognition characteristics of CKI, as discussed in the Introduction, it is of interest to survey the sequences surrounding the potential autophosphorylation sites between His^{317} and Pro^{342} (Fig. 5). None is preceded by a cluster of acidic residues, precluding this modality for recognition. The other motif recognized by CKI requires prior phosphorylation and so cannot account for the initial autophosphorylation. In any case, only one such site is available in this region: phosphorylation at Ser^{328} would create a potential site Xaa-Xaa-Yaa (where Yaa is not Ser or Thr) that could then act as a pseudosubstrate. Better understanding of the interactions between the catalytic and regulatory domains of CKI will have to await the solution of the three-dimensional structure of full-length CKI.

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