Casein Kinase II Catalyzes Tyrosine Phosphorylation of the Yeast Nucleolar Immunophilin Fpr3*

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In the yeast Saccharomyces cerevisiae, the nucleolar immunophilin, Fpr3, is phosphorylated at tyrosine and dephosphorylated by the phosphotyrosine-specific phosphoprotein phosphatase, Ptp1. In Ptp1-deficient cells, Fpr3 contains phospho-Tyr at a single site (Tyr184), but also contains phospho-Ser and phospho-Thr. Ser186 (adjacent to Tyr184) is situated within a canonical site for phosphorylation by casein kinase II (CKII). Yeast cell lysates contain an activity that binds to Fpr3 in vitro and phosphorylates Fpr3 at Ser, Thr, and Tyr; this activity was found to be dependent on expression of functional yeast CKII. Moreover, purified Fpr3 was phosphorylated on Tyr184 in vitro by either purified yeast CKII or purified, bacterially-expressed human CKII. Likewise, phosphorylation of Fpr3 at tyrosine in vivo was markedly enhanced in yeast cells overexpressing a heterologous (Drosophila) CKII, but was undetectable in yeast cells carrying only a temperature-sensitive allele of the endogenous CKII, even when the cells were grown at a permissive temperature. Phosphorylation of Fpr3 at Tyr184 by CKII in vitro lagged behind phosphorylation of Fpr3 at Ser, and was accelerated by pre-phosphorylation of Fpr3 at Ser using CKII. Furthermore, synthetic peptides corresponding to the sequence surrounding Tyr184 that contained P-Ser (or Glu) at position 186 were much more efficient substrates for CKII phosphorylation of Tyr184 than a synthetic peptide containing Ala at position 186. These findings indicate that CKII phosphor-ylates Fpr3 at tyrosine and serine both in vitro and in vivo and thus possesses dual specificity. These results also indicate that Tyr184 is phosphorylated by CKII via a two-step process, in which phosphorylation at the +2 position provides a negatively-charged specificity determinant that allows subsequent phosphorylation of Tyr184.

Protein kinases with dual specificity are able to phosphorylate either themselves and/or their target substrates at tyrosine and at serine or threonine (1). A number of protein kinases with dual specificity have been identified in budding yeast (Saccharomyces cerevisiae) and in fission yeast (S. pombe). These enzymes are likely to be responsible for all of the protein-bound phosphotyrosine found in these organisms, since no tyrosine-specific protein kinases have yet been detected in either yeast. Examples of such protein kinases in S. cerevisiae include: the mitogen-activated protein kinase kinase (MEK)2 homologs Ste7 (2), Pbs2 (3), Mkk1 and Mkk2 (4); a Wee1 homolog, Swel (5); the glycogen synthase kinase-3 homolog, Mck1 (6); and a protein kinase involved in DNA damage checkpoint control, Spk1/Rad53 (7). With one exception, all of the known phosphotyrosine-containing proteins in S. cerevisiae are themselves protein kinases, for example, the cell cycle kinase Cic28 (8) and the mitogen-activated protein kinases, Kas1 (9), and Fus3 (10). The exception is Fpr3, which is an abundant nucleolar protein that is a member of the FK506-binding subfamily of immunophilins with an as yet unknown physiological function (11). We have shown previously that phosphotyrosyl-Fpr3 is a cellular substrate of the S. cerevisiae protein-tyrosine phosphatase, Ptp1. This phosphatase directly dephosphorylates phosphotyrosyl-Fpr3 in vitro, and prevents accumulation of phosphotyrosyl-Fpr3 in vivo (12).

The phospho-Tyr that appears in Fpr3 in Ptp1-deficient cells is unlikely to result from autophosphorylation, because Fpr3 lacks homology to any known protein kinase and does not display detectable protein kinase activity in vitro. Hence, we sought to identify the protein kinase responsible for phosphorylating Fpr3 at Tyr. The COOH-terminal third of Fpr3 comprises its FK506-binding and peptidyl-prolyl-cis,trans-isomerase domain, whereas the NH2-terminal two-thirds of Fpr3 has homology to other nucleolar proteins, like nucleolin, and is responsible for localization of Fpr3 to the nucleolus (11). The NH2-terminal segment is phosphorylated at Ser, Thr, and Tyr, and in this region many of the potential phosphoacceptor residues are flanked by acidic residues. This arrangement suggested that Fpr3 might be a substrate for casein kinase II (CKII),2 because this enzyme has a requirement for acidic substrates (13) and because it is also localized to the nucleolus (14). Furthermore, we previously demonstrated that the exclusive site of tyrosine phosphorylation within Fpr3 is Tyr184 (underlined), which also resides in a highly acidic sequence (DEDEDADYDSEDYDLT) within the NH2-terminal domain (12). This observation raised the further possibility that phosphorylation of Fpr3 at Tyr might be catalyzed by CKII.

CKII is a well characterized, Ser/Thr-directed protein kinase with a broad range of reported substrates in vivo and with multiple roles in signal transduction (15, 16). CKII is composed

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1 L. Wilson, unpublished results.
2 The abbreviations used are: MEK, mitogen-activated protein kinase kinase; CKII, casein kinase II; CKI, casein kinase I; yCKII, yeast CKII; hCKII, human CKII; dCKII, Drosophila CKII; GST, glutathione S-transferase; Ptp1, protein-tyrosine phosphatase 1; PAGE, polyacrylamide gel electrophoresis.
of catalytic (α and/or α′) and regulatory (β and/or β′) subunits, associated as either an αββ2 or an αα′ββ′ tetramer. In S. cerevisiae, the α and α′ subunits of CKII are encoded by the CKA1 and CKA2 genes, respectively (17). Disruption of either gene still permits cell growth, whereas disruption of both genes is lethal. Yeast mutants which produce temperature-sensitive CKII undergo growth arrest within the time of about one cell cycle after shift to restrictive temperature (18). Half of the arrested cells are unbudded, and the other half have large buds, suggesting that CKII activity is required for progression through both G1 and G2/M.

Examination of natural protein substrates, and systematic studies using synthetic peptide substrates, indicate that the consensus sequence for CKII phosphorylation is Ser/Thr-X-X-Neg (where Neg indicates a negatively-charged residue or a residue carrying a negatively-charged modification), although acidic residues at positions spanning from −2 to +7 can act as positive specificity determinants for CKII, and an acidic residue at position +3 is not an absolute requirement (19). The order of efficacy of negatively-charged specificity determinants at the +3 position is thought to be: P-Tyr > P-Ser > Glu > P-Thr. Thus, phosphorylated Tyr and phosphorylated Ser surpass Glu, providing biochemical support for the possibility that CKII may participate in ordered multisite phosphorylation (20).

Here we demonstrate that phosphorylation of Fpr3 at Tyr, as well as at Ser and Thr, is dependent on CKII activity in vivo. Furthermore, we present evidence that CKII phosphorylates Tyr in vitro by a two-step mechanism involving prior phosphorylation of the Ser residue in the +2 position. These results indicate that CKII is not exclusively a Ser/Thr-directed protein kinase, but rather has dual specificity. Moreover, our results indicate that CKII has the previously unrecognized capacity to use P-Ser as a specificity determinant to permit subsequent phosphorylation of a nearby Tyr.

EXPERIMENTAL PROCEDURES

Yeast Strains—Standard methods were used for growth and transformation of yeast (21). Strains YDH6 and YDH8 were generous gift of C. Glover, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA) harbor disruptions of both CKA1 and CKA2; in strain YDH6, the CKII deficiency is complemented by a wild-type copy of CKA2, whereas in strain YDH8 the deficiency is complemented by a temperature-sensitive allele (cka2-8) carried on a CEN plasmid (18). To generate strains YDH6200 and YDH8200, the PTP1 gene in strains YDH6 and YDH8 was disrupted by homologous recombination using a 1.25-kilobase pair of 5′-AATGAACTGCTGCATGCCAAAC-3′ (22) and 5′-CCGATGCCGCTCGCTGGATAAT-3′ (23) were prepared, in which phosphoserine, glutamic acid, or alanine were substituted for Ser(126)(underlined). Peptide phosphorylation reactions (45 μl final volume) contained 1 mg/ml peptide, 1.5 μg/ml human CKII, 100 μM [γ-32P]ATP (5 Cimmol), 20 mM Tris-HCl (pH 7.2), 50 mM KCl, and 10 mM MgCl2. Samples (5 μl) were removed at intervals and mixed with 2 μl of 0.5 M EDTA to terminate the reaction. Reaction products were analyzed by spotting 3 μl of each sample onto 2 cm squares of DEAE-cellulose paper (NA45, Schleicher & Schuell, Keene, NH) and washing the paper for 5 min in each in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl. The papers were then rinsed in acetone and dried and the amount of radioactivity incorporated determined by counting in a liquid scintillation spectrometer.

Immunoprecipitation of Fpr3—Strains YDH6200 and YDH8200 were grown to stationary phase (A660nm = 4) and lysed by vigorous agitation with glass beads as described previously (11) in lysis buffer (100 mM NaCl, 50 mM Tris-HCl (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 12 mM benzamidine, 5 mM phenanthroline, 30 mM phenylmethylsulfonyl fluoride, 100 mM Na2VO4, and 0.5 μg/ml of each of the following protease inhibitors (aprotinin, leupeptin, soybean trypsin inhibitor, chymotrysin, and pepstatin). Lysates were clarified by centrifugation for 15 min at 10,000 × g, and the resulting supernatant fractions were centrifuged at 100,000 × g for 40 min. Proteins were eluted from the pellet by stirring the resuspended particulate material for 1 h at 4 °C in 0.5 ml of lysis buffer containing a final concentration of 1 mM NaCl. Insoluble particulate matter was removed from the suspension by centrifugation at 100,000 × g for 60 min. The resulting supernatant extract was diluted 6-fold in lysis buffer and incubated at 4 °C for 16 h with agarose beads to which had been coupled immunoglobulin from rabbits immunized against the amino terminus of Fpr3 (11). The beads were then washed 4 times in lysis buffer and solubilized by boiling in SDS-PAGE sample buffer. The immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described below.

Immunoblot and Phosphoamino Acid Analyses—Protein kinase reaction mixtures, samples of yeast lysates (100 μg), or solubilized immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblotting as described previously (12). The following antibodies (1 μg of protein/ml) were used as probes: anti-phosphotyrosine mAb 4G10 (24) (Upstate Biotechnology, Lake Placid, NY), immunoglobulin from rabbits immunized against the amino terminus of Fpr3 (11), or serum raised against Drosophila CKII holoenzyme (24) (generous gift of C. Glover).

Phosphoamino acid analyses were carried out as described previously (25). Briefly, proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore). Following autoradiography, the bands corresponding to protein of interest were cut out and subjected to acid hydrolysis. After addition of P-Tyr, P-Thr, and P-Ser, which served as both carriers and internal standards, the dried samples were resolved electrophoretically on thin-layer cellulose plates (Merck) at pH 1.9 in the first dimension and at pH 3.7 in the second dimension. Radiolabeled phosphoamino acids detected by autoradiography and unlabeled phosphoamino acids were detected by staining with ninhydrin.
RESULTS

Yeast Lysates Contain a CKII-dependent Activity That Phosphorylates Fpr3 at Ser, Thr, and Tyr—We have shown previously that yeast lysates contain an activity capable of binding to Fpr3 and of phosphorylating it in vitro at Ser, Thr, and Tyr (12). The resulting relative phosphoamino acid content is similar to that found in Fpr3 phosphorylated in vivo (phosphoserine, 85%; phosphothreonine, 11%; and phosphotyrosine, 4%) (12). To determine whether CKII is required for this phosphorylation, strain YDH6, a caa1 caa2Δ strain in which the CKII deficiency is complemented by wild-type CKA2, and strain YDH8, an isogenic strain in which the CKII deficiency is complemented by a temperature-sensitive allele (cka1-32; cka2-8) (18), were grown at the permissive temperature (25 °C), and cell lysates were prepared. These lysates were incubated with glutathione-Sepharose beads loaded with GST-Fpr3-N, a glutathione S-transferase fusion protein containing the NH2-terminal portion of Fpr3 (see “Experimental Procedures”). The beads were washed and incubated at 30 °C in a reaction buffer containing [γ-32P]ATP, and the reaction products analyzed by SDS-PAGE and autoradiography. Lysate from cells expressing wild-type CKA2 mediated efficient phosphorylation of GST-Fpr3-N (Fig. 1A, Lane 1), whereas lysate from cells expressing the cka2–8 temperature-sensitive mutant did not (Fig. 1A, Lane 2). These results indicate that the activity in yeast lysates that phosphorylates Fpr3 at serine, threonine, and tyrosine residues is CKII-dependent. YDH8 cells exhibit phenotypic changes characteristic of CKII depletion even at the permissive temperature and fail to grow at temperatures above 33 °C (18). Thus, the inability of lysates of YDH8 cells grown at the permissive temperature to phosphorylate Fpr3 probably reflects inactivation of the mutant CKII during incubation at 30 °C in vitro.

To examine the effect of CKII deficiency on phosphorylation of Fpr3 at tyrosine, kinase reactions similar to those described above were carried out using non-radioactive ATP, and the reaction products were examined by immunoblotting with anti-phosphotyrosine antibody (Fig. 1B). Incubation of GST-Fpr3-N with a lysate of YDH8 cells resulted in tyrosine phosphorylation of GST-Fpr3-N in the subsequent kinase reaction (Fig. 1B, Lane 1), whereas incubation with a lysate of the CKII-deficient YDH8 cells (Fig. 1B, Lane 2) or with buffer alone (Fig. 1B, Lane 4) did not. In addition, tyrosine phosphorylation was not detected when the Y184F mutant of GST-Fpr3-N (GST-Fpr3NY184F) was used as kinase substrate with the active lysate from YDH6 cells (Fig. 1B, Lane 3). Therefore, the kinase responsible for the tyrosine phosphorylation of Fpr3 in yeast lysates requires both the function of CKII and the integrity of the Tyr site in Fpr3 that is known to be phosphorylated in vivo.

Tyrosine Phosphorylation of Fpr3 in Vivo Requires Functional CKII—To enhance detection of phosphotyrosine in Fpr3 in vivo, derivatives of YDH6 and YDH8 lacking the PTP1 gene were generated by gene disruption as described previously (12). The resulting strains, YDH6200 and YDH8200, were grown to mid-exponential phase at the permissive temperature (25 °C). Cell lysates were subjected to immunoprecipitation with anti-Fpr3 antibody. Both lysates and immunoprecipitates were resolved by SDS-PAGE and the phosphotyrosyl proteins present were examined by immunoblotting with anti-phosphotyrosine antibody. Phosphotyrosyl-Fpr3 was detected in the lysate of YDH6200 cells (Fig. 2A, Lane 3), but not in the lysate of YDH8200 cells (Fig. 2A, Lane 2), even though immunoblot analysis of the same lysates using anti-Fpr3 antibody demonstrated that the total amount of Fpr3 protein was essentially the same in both extracts (Fig. 2A, Lanes 3 and 4). Likewise, Fpr3 immunoprecipitated from YDH6200 was phosphorylated at tyrosine, while Fpr3 immunoprecipitated from YDH8200 was not (Fig. 2B). These results indicate that, in vitro, tyrosine phosphorylation of Fpr3 in vivo is also CKII-dependent. It is interesting to note that an unidentified protein (~50 kDa) also appeared to be phosphorylated at tyrosine in a CKII-dependent manner (Fig. 2, Lane 1); this protein did not appear to be derived from Fpr3 because it did not react with anti-Fpr3 antibody.

In contrast to budding yeast CKII (an αβαβ heterotetramer encoded by four separate genes), Drosophila CKII (dCKII) is an ααββ tetramer and is encoded by only two genes, facilitating ectopic expression; moreover expression of dCKII can complement yeast cells lacking endogenous CKII (17). To determine if high-level expression of dCKII could affect the extent of tyrosine phosphorylation in Fpr3, a plasmid encoding the α and β subunits of dCKII under dual control of the divergent GAL1/10
promoter was introduced into ptp1 and PTPI strains. The resulting strains were grown in either galactose medium to induce expression of dCKII, or in glucose medium to repress dCKII expression. Gal-dependent production of dCKII was verified by immunoblot analysis of cell lysates with an antibody specific for dCKII (Fig. 3, lower panel). Immunoblot analysis using anti-Fpr3 antibodies indicated that the abundance of Fpr3 was not affected by expression of dCKII (data not shown). However, expression of dCKII resulted in enhanced tyrosine phosphorylation of Fpr3 in the ptp1 strain as detected by an increase in the signal obtained with the anti-phosphotyrosine antibody (Fig. 3, upper panel, Lane 2). This increase was not observed in the PTPI strain (Fig. 3, upper panel, Lane 3), indicating that the endogenous level of Ptp1 was sufficient to block the hyperphosphorylation of Fpr3 that resulted from dCKII expression.

CKII Directly Phosphorylates Fpr3 at Tyr184 in Vitro—The results described above suggest that CKII either directly phosphorylates Fpr3, or activates some other protein kinase which in turn phosphorylates Fpr3 at tyrosine. To determine whether CKII can directly phosphorylate Fpr3 at Tyr184, CKII heterotetramer purified to greater than 95% from S. cerevisiae (yCKII) was incubated with [γ-32P]ATP and, as substrate, either GST-Fpr3N or GST-Fpr3N(Y184F). The reaction products were resolved by SDS-PAGE and the phosphorylated fusion proteins were excised and subjected to phosphoamino acid analysis. yCKII phosphorylated Fpr3 at serine, threonine, and tyrosine (Fig. 4a, PAGE 1), the Fpr3 phosphorylated in vitro had a phosphoamino acid composition similar to that of Fpr3 phosphorylated in vivo (12). GST-Fpr3N(Y148F) was not phosphorylated at tyrosine, confirming that Tyr184 is the site of tyrosine phosphorylation (Panel A, PAGE 2). In further support of the conclusion that CKII itself, and not a contaminating kinase, was responsible for the tyrosine phosphorylation of Fpr3N, we found that human CKII (hCKII), expressed and purified from E. coli, also phosphorylated Fpr3 at Tyr184; phosphorysine was detected both by phosphoamino acid analysis of radiolabeled Fpr3N (Fig. 4, PAGE 1, Panel A, PAGE 3) and by anti-phosphotyrosine immunoblotting of Fpr3N phosphorylated by hCKII in the presence of non-radiolabeled ATP (Fig. 4, PAGE 1, Panel B). Auto-phosphorylation of CKII at Tyr was not detected. Because E. coli lacks detectable protein-tyrosine kinase activity (26, 27), these results provide strong evidence that CKII can directly phosphorylate Fpr3 at Tyr184 and hence that CKII is a protein kinase with dual specificity.

Phosphorylation of Fpr3 CKII Is an Ordered Process—To explore the mechanism of Tyr184 phosphorylation by CKII, we examined the kinetics of serine, threonine, and tyrosine phosphorylation of Fpr3 in vitro. hCKII was incubated with Fpr3N and [γ-32P]ATP, and the phosphoamino acid composition of Fpr3N was determined at various times during the reaction. Tyrosine phosphorylation occurred at a slower rate than either Ser or Thr phosphorylation and appeared biphasic (Fig. 5A). Tyrosine phosphorylation of Fpr3N reached a stoichiometry of 0.2 mol of P-Tyr/mol of Fpr3N, in agreement with our previous findings in vivo (12). One possible explanation for the unusual kinetics of incorporation into Tyr could be that prior serine or threonine phosphorylation is necessary for efficient tyrosine phosphorylation. To determine whether serine or threonine phosphorylation promotes Tyr184 phosphorylation, GST-Fpr3N was phosphorylated with CKII, then specifically dephosphorylated at Tyr184 by incubation with purified Ptp1; we have shown previously that this treatment removes all phospho-Tyr from Fpr3 without affecting its content of p-Ser and p-Thr (12). When pre-phosphorylated GST-Fpr3N was used as substrate for a second round of phosphorylation by CKII, the rate of incorporation into tyrosine was significantly greater than that observed with the unphosphorylated substrate (Fig. 5B). These results
Tyrosine phosphorylation of Fpr3 by CKII requires prior phosphorylation at serine. Panel A, kinetics of Fpr3N phosphorylation. Fpr3N was incubated with hCKII in the presence of [γ-32P]ATP (5 Ci/mmol). Samples were taken at intervals, resolved by SDS-PAGE, and the bands corresponding to Fpr3N were excised and subjected to phosphoamino acid analysis. Radioiodinated phosphoamino acids were quantified by liquid scintillation spectrometry. Values for phosphoserine (triangles), phosphothreonine (squares), and phosphotyrosine (circles) are expressed as the percent of final incorporation and represent the mean of duplicate experiments; error bars represent standard deviations. Panel B, effect of prior serine phosphorylation on tyrosine phosphorylation of Fpr3. GST-Fpr3N beads were incubated with hCKII for 1 h in the presence (triangles) or absence (circles) of ATP, then treated with Ptp1 to dephosphorylate Tyr184. The resulting serine-phosphorylated and mock-phosphorylated GST-Fpr3N beads were incubated with hCKII and nonradioactive ATP. The reactions were sampled at intervals, and GST-Fpr3N was resolved by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibody. Relative values were obtained by scanning densitometry. Panel C, tyrosine phosphorylation of synthetic peptides. Three peptides corresponding to the tyrosine-phosphorylated region of Fpr3 with alterations at Ser186 (underlined) (DEDADYDSD/EDYDL (triangles), DEDADYD/DESYDL (squares), and DEDADYD/DESYDL (circles) were incubated with hCKII and [γ-32P]ATP. The reaction mixtures were sampled at intervals, and total incorporation was quantitated using DEAE-cellulose papers as described under "Materials and Methods."  

The results presented here indicate that the yeast immunophillin Fpr3 can be phosphorylated at tyrosine by CKII from a variety of sources. Thus CKII exhibits the properties of a protein kinase with dual specificity. The site phosphorylated in Fpr3 is Tyr184, and this residue is phosphorylated both in vitro and in vivo. Phosphorylation at Tyr184 appears to occur via a processive mechanism, in which prior phosphorylation at Ser186 is required for subsequent phosphorylation at Tyr184.

Previous studies in vitro have led to the conclusion that CKII phosphorylates substrates only at serine and threonine residues (15, 16); CKII is not capable of phosphorylating the Tyr-containing peptides RRREEEYEEE or DAEEYARRG (28, 29). More recently, CKII purified from Yarrowia lipolytica was reported to be capable of low levels of tyrosine autophosphorylation and of poly-Glu:Tyr phosphorylation (30), although the possible contribution of a contaminating kinase was not addressed. Indeed, in our hands bacterially-purified recombinant hCKII and CKII purified from S. cerevisiae did not display these activities. However, we found, as reported here, that CKII could phosphorylate the Fpr3 peptide DEDADYD-S/P/EDYDL at Tyr184 (underlined), the site at which Fpr3 undergoes CKII-dependent tyrosine phosphorylation in vivo. These findings suggest that Tyr phosphorylation by CKII is substrate-specific, and that it occurs preferentially at sequences similar to those surrounding Tyr184 in Fpr3. It is unclear at present whether tyrosine phosphorylation of Fpr3 by CKII is an event unique to this substrate, or whether CKII also phosphorylates other proteins containing tyrosine residues within the required sequence motifs. In this regard, although there is no direct evidence that CKII phosphorylates proteins other than Fpr3 at tyrosine, we observed at least one other protein in Ptp1-deficient yeast that appeared to be phosphorylated at tyrosine in a CKII-dependent manner.

There are now a number of examples of processive or hierarchical multisite protein phosphorylation. In many cases, the phosphorylation of a serine residue by a primary protein kinase is a prerequisite for subsequent phosphorylation by a second protein kinase. Because the second protein kinase is usually acidotropic, the primary phosphorylation may function to acidify the amino acid environment of the second phosphoacceptor site (reviewed in (31)). For example, glycogen synthase kinase-3, which has a minimal substrate recognition sequence of SXXS(P), phosphorylates glycogen synthase at 4 serine residues only after phosphorylation at a primary serine by...
CKII (32). Using peptide substrates to dissect the mechanism of glycogen synthase kinase-3-catalyzed multisite phosphorylation, glycogen synthase kinase-3 was shown to phosphorylate a series of serines in an obligate order with the sequential formation of new recognition sequences (33). Glycogen synthase is also phosphorylated at serine by cAMP-dependent protein kinase; this phosphorylation occurs at position –3 relative to a second serine, which can then be phosphorylated by another acidotropic protein kinase, casein kinase I (34, 35).

The insulin receptor, which autoprophosphorylates at three successive tyrosine residues in an obligate order, provides an example of hierarchical multisite phosphorylation at tyrosine residues (36).

Phosphorylation of Ser186 in Fpr3 appears to be a specificity determinant for the phosphorylation of Tyr184 by CKII. Although studies on phosphorylation of peptides have shown that CKII can utilize phosphoaminos acids as specificity determinants in vitro (20, 37, 38), to our knowledge, our results represent the first report to implicate CKII as a secondary kinase in hierarchical phosphorylation at Tyr in vivo. In peptides corresponding to the phosphorylated region of Fpr3, substitution of Ser186 with either (P)Ser186 or Glu, but not with Ala, allowed CKII-catalyzed phosphorylation of Tyr184, suggesting that an acidic residue at the +2 position is critical, even though in this peptide the amino acids at positions +1, +3, and +4 are also acidic. This specificity is in apparent contrast to serine/threonine phosphorylation by CKII, which is not heavily dependent upon the presence of an acidic residue at the +2 position, provided that there is an acidic residue at +3 (13). We observed a slower rate of phosphorylation at Tyr versus Ser for previously unphosphorylated Fpr3, and an enhanced rate of tyrosine phosphorylation using as a substrate Fpr3 that was previously phosphorylated at Ser. These kinetics can be explained by the requirement for prior phosphorylation at Ser186, although another factor which might also contribute to the delay is that unphosphorylated serines (including Ser186) might be phosphorylated preferentially over Tyr184, acting as competitive inhibitors of CKII.

The structural basis for the dual specificity of CKII is not obvious. The division between serine/threonine-specific and tyrosine-specific protein kinases has narrowed in recent years, as a number of protein kinases previously considered exclusively serine/threonine-specific have been shown to display dual specificity. For example, CKII autophosphorylates at tyrosine, and is able to phosphorylate certain artificial substrates at tyrosine in vitro (39). The substrate specificity of phosphatase kinase is determined by davalent cations; Mg2+ promotes serine phosphorylation of phosphoprotein b, whereas Mn2+ promotes tyrosine phosphorylation of angiotensin II (40). Glycogen synthase kinase-3β autoprophosphorylates at tyrosine, resulting in an increase in its serine/threonine transphosphorylation activity (41). In these cases, and in the case of CKII, tyrosine phosphorylation appears to be restricted to a small number of substrates and/or is less efficient than serine/threonine phosphorylation by the same kinases. In fact, few protein kinases with dual specificity display equal activity toward both residues (the MEKs being the best characterized examples). The structural constraints of the catalytic domain are such that it may be difficult to achieve an intermediate structure equally efficient at using as phosphoacceptors both alkyl and aromatic hydroxyls (42).

The possible effects of tyrosine phosphorylation by CKII are also unknown. While Tyr184 in the context of a peptide is phosphorylated to completion by CKII, it is only phosphorylated to approximately 20% stoichiometry in the context of the whole protein in vivo and in vitro (Ref. 12 and this report). This could be due to structural constraints imposed by the rest of the protein. As noted above, tyrosine phosphorylation by CKII appears to be less efficient than serine/threonine phosphorylation. However, the low stoichiometry of the phosphorylation of Fpr3 at Tyr184 does not necessarily preclude the possibility that Tyr184 phosphorylation is functionally significant in vivo. Preliminary experiments indicate that Fpr3 tyrosine phosphorylation increases as cells enter stationary phase, suggesting that tyrosine phosphorylation by CKII may be a regulated process. We have been unable to determine whether Fpr3 function is affected by tyrosine phosphorylation, both because of the lack of physiological assays for Fpr3 function, and because only ~20% of Fpr3 is tyrosine phosphorylated even in ptp1 yeast (12). Since we now know that CKII is the enzyme responsible for this modification, it may be possible to generate Fpr3 stochiometrically phosphorylated at Tyr184 and thus determine the effects of this phosphorylation on Fpr3 function. At present we cannot rule out the alternative possibility that CKII and perhaps other dual-specific protein kinases might phosphorylate tyrosine residues in an adventitious manner. In this case, the role of phosphotyrosine-specific phosphatases, such as Ptp1, would be to reverse such adventitious tyrosine phosphorylation. Further characterization of the factors that control tyrosine phosphorylation by CKII will be necessary to determine if this aspect of its activity has an important physiological function.

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REFERENCES
1. Lindberg, R. A., Quinn, A. M., and Hunter, T. (1992) Trends Biochem. 17, 114–119
2. Errede, B., and Levin, D. E. (1993) Curr. Opin. Cell Biol. 5, 254–260
3. Brewster, J. L., devaletur, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) Science 260, 1760–1763
4. Irie, K., Takase, M., Lee, K. S., Levin, D. E., Arakii, H., Matsumoto, K., and Oshima, Y. (1993) Mol. Cell. Biol. 13, 3076–3083
5. Bocher, R. N., Deshaies, R. J., and Kirschner, M. W. (1993) EMBO J. 12, 3417–3426
6. Lim, M.-Y., Dailey, D., Martin, G. S., and Thorner, J. (1995) J. Biol. Chem. 268, 21155–21164
7. Sun, Z., Fay, D. S., Marini, F., Fioami, and Stern, D. F. (1996) Genes Dev. 10, 395–406
8. Surber, P. K., and Murray, A. W. (1992) Nature 355, 365–368
9. Mad, D., Cook, J. G., and Thorner, J. (1995) Mol. Biol. Cell 6, 889–909
10. Gartner, A., Nasmyth, K., and Ammerer, G. (1992) Genes Dev. 6, 1280–1292
11. Benton, B., Zang, J.-H., and Thorner, J. (1994) J. Cell Biol. 127, 623–639
12. Wilson, L. K., Benton, B. M., Zhou, S., Thorner, J., and Martin, G. S. (1995) Mol. Biol. Cell 6, 270, 9136–9140
13. Tuazon, T. P., and Traugh, J. A. (1991) Adv. Second Messenger Phosphoprotein Res. 23, 123–165
14. Paff, M., and Anderer, P. F. (1988) Biochim. Biophys. Acta 969, 100–109
15. Allende, J. W., and Allende, C. C. (1995) PASE FB J. 9, 313–323
16. Litchfield, D. W., and Lusher, B. (1993) Mol. Cell. Biochem. 127/128, 187–199
17. Padmanabha, R., Chen-Wu, J. L-P., Hanna, D. E., and Glover, C. V. C. (1990) Mol. Cell. Biol. 10, 4089–4099
18. Hanna, D. E., Reithinawamy, and Glover, C. V. C. (1995) J. Biochem. 270, 5995–59914
19. Meggio, F., Marin, O., and Pinna, L. A. (1994) Cell. Mol. Biol. Res. 40, 401–409
20. Meggio, F., Perich, J. W., Reynolds, E. C., and Pinna, L. A. (1993) FEBS Lett. 279, 307–309
21. Anzuelo, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) Current Protocols in Molecular Biology, Wiley-Interscience, New York
22. Bidwai, A. P., Reed, J. C., and Glover, C. V. C. (1994) Arch. Biochem. Biophys. 309, 348–355
23. Drucker, B. J., Mamont, H. J., and Roberts, T. M. (1989) N. Engl. J. Med. 321, 1383–1391
24. Dahmus, G. K., Glover, C. V. C., Bruttal, D. L., and Dahmus, C. E. (1984) J. Biol. Chem. 259, 9001–9006
25. Boyle, W. J., Geer, P. V. D., and Hunter, T. (1991) Methods Enzymol. 201, 110–149
26. Wang, J. Y. J., and Baltimore, D. (1985) J. Biol. Chem. 260, 64–71
27. Foster, R. S., Thorner, J., and Martin, G. S. (1989) J. Bacteriol. 171, 272–279
28. Kuenzel, E. A., Mulligan, J. A., Sommerson, J., and Krebs, E. G. (1987) J. Biol. Chem. 262, 9136–9140
29. Marin, O., Meggio, F., Marchiori, F., Bovin, G., and Pinna, L. A. (1986) Eur. J. Biochem. 160, 239–244
30. Chardot, T., Shen, H., and Meunier, J.-C. (1995) C. R. Acad. Sci. Paris Life Sci.
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31. Roach, P. J. (1991) J. Biol. Chem. 266, 14139–14142
32. Roach, P. J. (1990) FASEB J. 4, 2961–2968
33. Fiol, C. J., Wang, A., Roeske, R. W., and Roach, P. J. (1990) J. Biol. Chem. 265, 6061–6065
34. Flotow, H., Graves, P. R., Wang, A., Fiol, C. J., Roeske, R. W., and Roach, P. J. (1990) J. Biol. Chem. 265, 14264–14269
35. Huang, K.-P., Akatsuka, A., Singh, T. J., and Blake, K. R. (1983) J. Biol. Chem. 258, 7094–7101
36. Levine, B. A., Clock, B., and Ellis, L. (1991) J. Biol. Chem. 266, 3565–3570
37. Hrubey, T. W., and Roach, P. J. (1990) Biophys. Biochem. Res. Commun. 172, 190–196
38. Litchfield, D. W., Arendt, A., Lozeman, F. J., Krebs, E. G., Hargrave, P. A., and Palczewski, K. P. (1990) FEBS Lett. 261, 117–120
39. Hoekstra, M. F., Dhillon, N., Carmel, G., DeMaggio, A. J., Lindberg, R. A., Hunter, T., and Kuret, J. (1994) Mol. Biol. Cell 5, 877–886
40. Yuan, C.-J., Huang, C.-Y. F., and Graves, D. J. (1993) J. Biol. Chem. 268, 17053–17056
41. Wang, Q. M., Fiol, C. J., DePaoli-Roach, A. A., and Roach, P. J. (1994) J. Biol. Chem. 269, 14566–14574
42. Taylor, S. S., Radzio-Andelm, E., and Hunter, T. (1995) FASEB J. 9, 1255–1266