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Budding and Fission Yeast Casein Kinase I Isoforms Have Dual-specificity Protein Kinase Activity

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We have examined the activity and substrate specificity of the *Saccharomyces cerevisiae* Hrr25p and the *Schizosaccharomyces pombe* Hhp1, Hhp2, and Cki1 protein kinase isoforms. These four gene products are isotypes of casein kinase I (CKI), and the sequence of these protein kinases predicts that they are protein serine/threonine kinases. However, each of these four protein kinases, when expressed in *Escherichia coli* in an active form, was recognized by anti-phosphotyrosine antibodies. Phosphoamino acid analysis of ^{32}P-labeled proteins showed phosphorylation on serine, threonine, and tyrosine residues. The *E. coli* produced forms of Hhp1, Hhp2, and Cki1 were autophosphorylated on tyrosine, and both Hhp1 and Hhp2 were capable of phosphorylating the tyrosine-protein kinase synthetic peptide substrate polymer poly-E_4Y_1. Immune complex protein kinases assays from *S. pombe* cells showed that Hhp1-containing precipitates were associated with a protein-tyrosine kinase activity, and the Hhp1 present in these immunoprecipitates was phosphorylated on tyrosine residues. Although dephosphorylation of Hhp1 and Hhp2 by Ser/Thr phosphatase had little effect on the specific activity, tyrosine dephosphorylation of Hhp1 and Hhp2 caused a 1.8-to 3.1-fold increase in the Km for poly-E_4Y_1 and casein. These data demonstrate that four different CKI isoforms from two different yeasts are capable of protein-tyrosine kinase activity and encode dual-specificity protein kinases.

INTRODUCTION

Casein kinase I (CKI) is a serine/threonine protein kinase activity that is detected in all eukaryotic cells (reviewed in Tuazon and Traugh, 1991). Unlike most other protein kinases, CKI is acidotrophic, preferring acidic residues in its substrates (Flotow and Roach, 1991). Substrates containing the motif Ser/Thr(PO_4)-Xaa-Xaa-Ser/Thr also are recognized by CKI (Flotow *et al.*, 1990), suggesting that prior phosphorylation can direct substrate specificity in vivo. This links the activity of CKI in a hierarchical protein phosphorylation relay whereby CKI can amplify or dampen signal transduction and allows for the recruitment of CKI into second messenger-dependent phosphorylation cascades (Roach, 1991).

The sizes of CKI activities range from 25 to 60 kDa, but CKI has been most frequently described as a single ~30-kDa subunit (Tuazon and Traugh, 1991). The broad size distribution for CKI activity is most easily explained by the existence of multiple isoforms encoded by distinct genes and by alternative splice variants. This notion has been confirmed by the cloning and analysis of CKI cDNAs from several eukaryotic species that encode polypeptides ranging between 34 and 62 kDa. *Saccharomyces cerevisiae* has four different genes, *HRR25*, *CKI1*, *CKI2*, and *CKI3* (Hoekstra *et al.*, 1991; Robinson *et al.*, 1992; Wang *et al.*, 1992; Hoekstra, Dhillon, and DeMaggio, unpublished data) that encode proteins with molecular weight between 55 and 62 kDa.

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Schizosaccharomyces pombe has at least four CKI genes, *hhp1*$^+$, *hhp2*$^+$, *cki1*$^+$, and *cki2*$^+$ (Dhillon and Hoekstra; 1994, Wang *et al.*; 1994b), and the proteins encoded by these genes range from 45 to 50 kDa. In vertebrates, four closely related CKI isoforms (α, β, γ, δ) have been reported and these range from 34 to 49 kDa (Rowles *et al.*, 1991; Zhai *et al.*, 1992; Graves *et al.*, 1993).

Several studies suggest where CKI might play an important role in cellular metabolism and regulation. CKI shows cell cycle–dependent localization to mitotic spindles (Brockman *et al.*, 1992), implicating a role for the enzyme in mitosis. Also, a CKI-like activity can be stimulated by insulin in a dose-dependent fashion and by IL-1 or tumor necrosis factor (Cobb and Rosen, 1983; Guy *et al.*, 1991; Guesdon *et al.*, 1993), and membrane-associated CKI activity can be inhibited by phosphatidylinositol 4,5-bisphosphate (Brockman and Anderson, 1991). Furthermore, sites phosphorylated by CKI both in vivo and in vitro have been determined for cAMP responsive element modulator (CREM) (de Groot *et al.*, 1993), SV40 large T antigen (Cegielska and Virshup, 1993), glycogen synthase (Roach, 1991), and p53 (Milne *et al.*, 1992). In CREM, the phosphorylation by CKI affects the DNA-binding activity of the transcription factor. For SV40, the residues phosphorylated by CKI are important for T antigen-driven replication capacity. In glycogen synthase, CKI phosphorylation is involved in inhibiting enzyme activity. For p53, the role of CKI phosphorylation in regulating p53 activity is not understood.

The majority of eukaryotic protein kinases have been classified into two groups based on their specificity for the hydroxyamino acid phosphorylated in substrate proteins. One class, the protein serine/threonine kinases, modifies either Ser, Thr, or both. The other class, the protein-tyrosine kinases, phosphorylate Tyr. Recently, a number of protein kinases that phosphorylate both at Tyr and at Ser/Thr (so called "dual-specificity" enzymes) have been described (Lindberg *et al.*, 1992). In *S. cerevisiae*, examples of these enzymes include Mck1p and Spk1p (Dailey *et al.*, 1990, Stern *et al.*, 1991; Lim *et al.*, 1993, Zheng *et al.*, 1993). Although the gene structure and classical characterization of CKI activity has revealed that CKI is most closely related to serine/threonine protein kinases, several experiments have suggested that CKI might have a promiscuous substrate specificity. A preparation of CKI from human erythrocytes was found to catalyze the phosphorylation of protein tyrosine in a variety of substrates such as angiotensin-II, tyrosine-containing peptides, alkylated bovine serum albumin (BSA), band 3, and ankyrin (Lu and Tao, 1986).

The recent identification of CKI-encoding genes in *S. cerevisiae* and *S. pombe* has allowed an examination of the significance of CKI activity. In this report we extend the biochemical description of yeast CKI (DeMaggio *et al.*, 1992; Vancura *et al.*, 1993; Carmel *et al.*, 1994) and report a novel characteristic of *S. cerevisiae* and *S. pombe* forms of CKI, namely the ability to phosphorylate serine, threonine, and tyrosine residues.

MATERIALS AND METHODS

Materials

Antiphosphotyrosine antibodies were monoclonal antibody 4G10 from Upstate Biotechnology (Lake Placid, NY) or rabbit polyclonal anti-phosphotyrosine serum (Kamps and Sefton, 1988) kindly provided by Bart Sefton (The Salk Institute, San Diego, CA). Recombinant protein phosphatases were the generous gift of David Barford (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). *Escherichia coli* expression plasmids pRSET::HRR25 and pRSET::hrr25-KR38 contain *HRR25* fused at its translation initiation codon to the bacteriophage T7 gene 10 initiation codon. Details of the construction of these plasmids are outlined in DeMaggio *et al.* (1992). Expression of the *hhp1*$^+$ gene in *E. coli* for the experiments shown in Figure 2 used the T7 gene 10-based expression plasmid pRSET6B (Invitrogen, La Jolla, CA). This construct and the HA epitope-tagged construct were described in Dhillon and Hoekstra (1994). In vivo labeling of Hrr25p and Hhp1, as shown in Figures 1 and 2, exactly followed the conditions and methods described in DeMaggio *et al.* (1992) and Lindberg *et al.* (1993). Anti-phosphotyrosine antibody probing of *E. coli* lysates was as described by Lindberg *et al.* (1993), and the in vivo labeling of Hrr25p was identical to that shown in DeMaggio *et al.* (1992). For experiments using rabbit anti-phosphotyrosine antibody, Western blots were probed as described by Kamps and Sefton (1988).

For protein overproduction, the cDNAs for *hhp1*$^+$ and *hhp2*$^+$ were modified by adding useful restriction sites (*Nde* I at the initiation codon and *Xho* I just after the termination codon) using polymerase chain reaction (PCR) as described previously (Wang *et al.*, 1992). The resulting PCR fragments were digested with *Nde* I/*Xho* I and ligated into the *Nde* I/*Xho* I sites of expression vector pET-15b. This derivative of the T7 expression system (Studier *et al.*, 1990) drives the overproduction of proteins fused to an N-terminal, 20-residue peptide (Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-) that allows affinity purification on immobilized nickel columns (Hochuli *et al.*, 1987). The final constructs (pET-15b/hhp1, pET-15b/hhp2) were transformed into BL21(DE3) cells to create the strains used for protein overproduction. Expression and purification of poly-His tagged CKI were as described by Carmel *et al.* (1994).

E. coli cells containing the pET-15b-based plasmids were grown in LB broth containing 200 μg/ml ampicillin at 37°C to an A_{600nm} of 1.0, at which point isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 3 h of induction, cells were harvested by centrifugation (20 min at 3000 × *g*, 4°C), washed with STE buffer (10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA), and stored at −70°C until used. A 3-l growth typically yielded 8 g (wet weight) of cells.

Purification of Polyhistidine-tagged Hhp1 and Hhp2

All steps were carried out at 4°C and were identical for Hhp1 and Hhp2. Frozen cells were thawed, resuspended in 5 vol of lysis buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 5 mM imidazole, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 5 μg/ml each of leupeptin, aprotinin, and pepstatin), and ruptured by two passes through a French press operated at 1000 psi. The resulting homogenate was sonicated briefly (=15 s) to shear nucleic acids, made 0.1% Brij 35, and centrifuged (100 000 × *g*, 1 h) to yield a clear supernatant (crude extract).

After filtration through a 0.45-μm filter, the extract was loaded directly onto a 4-ml Ni^{2+}-nitrolotriacetate-agarose column preequilibrated in lysis buffer containing 0.1% Brij 35. The column was washed with 200 ml (50 bed volumes) of lysis buffer containing 0.05% Brij 35 and was developed with sequential 20-ml steps of lysis buffer containing 0.02% Brij 35 and 10, 20, 40, and 60 mM imidazole. Frac-

tions containing casein kinase activity (eluting at 40 mM imidazole) were pooled, brought to 75% saturation with solid $(NH_4)_2SO_4$, stirred 20 min, and then centrifuged 20 min at 27 000 × *g*. The resulting pellet was resuspended in Buffer A (10 mM *N*-2-hydroxyethylpiperazine-N¹-2-ethanesulfonic acid [HEPES] pH 7.5, 250 mM NaCl, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid [EGTA]) to a final volume of 4 ml and loaded directly onto a 180-ml column (1.6 × 91 cm) of Sephacryl S-100 HR gel filtration medium equilibrated and run in Buffer A at 30 ml/h. Fractions containing casein kinase activity were pooled, diluted fivefold with 0.02% Brij 35 (to reduce the NaCl concentration to 50 mM), and loaded onto a MonoQ 5/5 high-pressure liquid chromatography column equilibrated in Buffer B (10 mM Tris-HCl pH 7.5, 0.02% Brij 35, 0.1% 2-mercaptoethanol) containing 50 mM NaCl. The gradient was washed with 5 bed volumes of this buffer and developed with a 20 ml linear gradient of increasing NaCl (from 50 to 400 mM). Fractions containing casein kinase activity were pooled, concentrated by dialysis against storage buffer (50% glycerol, 10 mM 3-(*N*-morpholino) propanesulfonic acid [MOPS] pH 7.0, 250 mM NaCl, 0.1 mM EGTA, 0.02% Brij 35, 1 mM dithiothreitol [DTT]), and stored at −20°C.

Phosphoamino Acid Analysis

Phosphoamino acid analysis using acid hydrolysis were carried out on proteins bound to Immobilon as described by Lindberg *et al.* (1993). High voltage, two-dimensional, thin-layer electrophoresis of hydrolyzed phosphoprotein samples was performed as described by Boyle *et al.* (1991).

Analysis of Hhp1 from S. pombe

Native protein lysates were prepared from logarithmically growing cultures (at an approximate cell density of 5×10^6 cells ml^{-1}) of *S. pombe* in synthetic media. Briefly, 1×10^8 cells were resuspended in 20 μl of lysis buffer (25 mM MOPS pH 7.2, 60 mM β-glycerolphosphate, 15 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 0.1 mM Na_3VO_4, 1% Triton X-100, 1 mM PMSF, 20 μ ml^{-1} leupeptin, and 2% Trasylol [Miles, Kankakee, IL]) and 5 vol of acid-washed glass beads were added to the cell suspension. Cells were vortexed five to six times for 30 sec at 2-min intervals, and the lysate was separated from the glass beads by piercing a hole through the microcentrifuge tube followed by a brief centrifugation into another microcentrifuge tube at 100 × *g* for 1 min. Lysates were cleared at 16 000 × *g* for 10 min at 4°C. Supernatants were assayed for total protein content using Bradford's reagent, and equivalent amounts of protein from each strain were used for immunoprecipitation reactions. Pellets from the clearing spin were resuspended in a small volume (20 μl) of boiling buffer (10 mM Tris-HCl pH 8.0, 1% sodium dodecyl sulfate [SDS]) and heated to 100°C for 3 min. The volume was brought up to 0.5 ml with lysis buffer, and this fraction, designated as the pellet fraction, was used for subsequent immunoprecipitation reactions and Western blot analysis.

Immunoprecipitation reactions were performed in a reaction volume of 0.5 ml in lysis buffer. Five micrograms of affinity-purified monoclonal antibody (12CA5) against the HA epitope (BabCo, Berkley, CA) were incubated with ~0.25–0.5 mg of total protein on ice for 60 min, followed by the addition of rabbit anti-mouse antisera (5 μg) for a further 45 min. Reactions were precipitated with protein A agarose (Repligen, Minneapolis, MN) with end-over-end shaking for 60 min at 4°C. The immunoprecipitates were washed six times in 1.0 ml of lysis buffer, and the protein A pellets were boiled in an equal volume of SDS loading buffer for SDS-polyacrylamide gel electrophoresis (PAGE). For in vitro kinase assays, immunoprecipitates were incubated in 1× kinase buffer (50 mM Tris-HCl pH 7.5, 12 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT) and [γ-^{32}P]ATP at a final concentration of 1 μM at 30°C for 10 min before boiling in SDS loading buffer and electrophoresis.

Dephosphorylation Reactions

To dephosphorylate phosphotyrosine residues, purified Hhp1 and Hhp2 were incubated at 37°C for 30 min with recombinant T-cell protein tyrosine phosphatase (500:1 wt/wt) in T cell protein-tyrosine phosphatase (PTPase) buffer (10 mM Tris-HCl pH 7.5, 0.1% 2-mercaptoethanol, 0.02% Brij 35). Reactions were terminated by the addition of an equal volume of 2 mM Na_3VO_4. To dephosphorylate phosphoserine and phosphothreonine residues, purified Hhp1 and Hhp2 were incubated at 37°C for 30 min with recombinant phosphoprotein phosphatase 1 (5:1 wt/wt) in PrP buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5 mM $MnCl_2$, 0.1% 2-mercaptoethanol, 0.02% Brij 35). Reactions were terminated by the addition of an equal volume of 0.6 mM okadaic acid (OA). Terminated reactions were subjected to SDS-gel electrophoresis or phosphotransferase assay as described below.

Autophosphorylation of Hhp1 on Tyrosine

Purified Hhp1 was dephosphorylated by PTPase treatment as described above and diluted into phosphotransferase buffer (25 mM 2-(*N*-morpholino) ethanesulfonic acid [MES] pH 6.5, 50 mM NaCl, 15 mM $MgCl_2$, 2 mM EGTA) containing 1 mM Na_3VO_4. Autophosphorylation was initiated at 37°C by the addition of ATP to a final concentration of 25 μM. The time course of tyrosine autophosphorylation (25 μg/ml Hhp1) was determined by removing aliquots (100 ng Hhp1) at 0, 5, 10, 15, and 30 min for phosphotyrosine quantitation (described below). The concentration dependence of tyrosine autophosphorylation was determined by incubating various concentrations of Hhp1 (5, 10, 25, and 50 μg/ml) for 15 min. Phosphotyrosine content was determined on 10-ng samples as described below.

Western Assay for Phosphotyrosine

Autophosphorylation reactions were terminated by boiling (5 min) in the presence of SDS sample buffer (3% SDS, 50 mM Tris-HCl pH 6.8, 8% glycerol, 2% 2-mercaptoethanol). Samples (10–100 ng Hhp1) were electrophoresed through 10% polyacrylamide gels and transferred to nitrocellulose membranes as described previously (Wang *et al.*, 1992). Phosphotyrosine was labeled with monoclonal antibody 4G10 (1 μg/ml) and detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) using sheep anti-mouse IgG conjugated to horseradish peroxidase. Images were collected on X-ray film and quantified in arbitrary absorbance units by laser densitometry (Molecular Dynamics, Sunnyvale, CA).

Analytical Methods

Phosphotransferase assays with casein or poly-E_4Y_1 as protein substrate were performed as described previously (Racker, 1991; Vancura *et al.*, 1993). Initial velocity measurements were carried out in duplicate with casein (0.45, 0.6, 1.0, 2.0, and 4.0 mg/ml) or poly-E_4Y_1 (10, 25, 100, 200, 500 μg/ml) as the varied substrate. When necessary, the phosphatase inhibitors Na_3VO_4 (1 mM) or OA (0.3 mM) were included. Estimates of Km, Vmax, and their SE were calculated as described by Wilkinson (1961). Protein-bound phosphate was determined in triplicate as described by Carmel *et al.* (1994) and reported as mean ±SD. SDS-polyacrylamide gels were prepared as described previously (Vancura *et al.*, 1993). Molecular mass markers included BSA (66.2 kDa), ovalbumin (42.7 kDa), bovine carbonic anhydrase (29.0 kDa), and α-lactoglobulin (18.4 kDa).

The percentage of CKI molecules containing phosphotyrosine was estimated after immunoprecipitation with antiphosphotyrosine antibody 4G10. A constant amount of protein kinase (160 nM) was incubated with increasing amounts of 4G10 (0, 0.08, 0.16, 0.8, 1.6 μM) in 20 μl on ice for 1 h and precipitated with an equal volume of protein-A agarose (50% slurry). Kinase activity remaining in the supernatant was assayed in duplicate as described above. The resultant data was fit to a binding isotherm to calculate the maximum amount of immunoprecipitable kinase ± SE of the estimate.

RESULTS

Baker's Yeast Hrr25p Is a Dual-specificity Protein Kinase

To characterize the substrate specificity of CKI, selected yeast isoforms of the enzyme were expressed in *E. coli* and assayed for the presence of phosphotyrosine. The goal was to determine whether the observation by Lu and Tao (1986) that erythrocyte CKI contains tyrosine kinase activity could be extended to recombinant yeast isoforms of CKI. *E. coli* was used as a host for these experiments because many CKI isoforms are efficiently expressed in bacteria (DeMaggio *et al.*, 1992; Zhai *et al.*, 1992; Graves *et al.*, 1993; Carmel *et al.*, 1994), and because *E. coli* contains little if any endogenous protein tyrosine kinase activity (Lindberg *et al.*, 1993).

First, we examined the ability of Hrr25p, a budding yeast CKI isoform, to react with an anti-phosphotyrosine antibody. As shown in Figure 1, extracts containing fully active, wild-type Hrr25p show a strong anti-phosphotyrosine immunoreactivity centered around 60 kDa. Conversely, this immunoreactivity was not observed in control extracts containing either catalytically inactive Hrr25p-K38R (Figure 1) (DeMaggio *et al.*, 1992) or the catalytic subunit of the mammalian cAMP-dependent protein kinase. In parallel comparisons, the anti-phosphotyrosine immunoreactive staining is as robust as that observed with *E. coli* extracts containing the PYT dual-specificity protein kinase or LYN tyrosine protein kinases (Lindberg *et al.*, 1993), indicating that the anti-phosphotyrosine reactivity is specific to extracts containing active Hrr25p. We conclude that Hrr25p contains phosphotyrosine residues as a result of autophosphorylation.

Second, to confirm the presence of phosphotyrosine, ^{32}P-radiolabelled *E. coli* protein extracts prepared from transformants containing either *HRR25, hrr25-K38R,* or vector alone were fractionated by gel electrophoresis, transferred to membranes, and subjected to autoradiography. As shown in Figure 1, wild-type Hrr25p, but not Hrr25p-K38R or the vector control, catalyzed the transfer of phosphate to endogenous *E. coli* proteins in addition to autophosphorylation. Phosphoamino acid analysis performed on autophosphorylated Hrr25p confirmed the presence of phosphotyrosine that, by Cerenkov counting, comprised 10% of the total ^{32}P bound to Hrr25p (Figure 1).

To determine if Hrr25p could phosphorylate substrates other than itself on tyrosine, four radiolabeled proteins, including Hrr25p, were subjected to phosphoamino acid analysis (Figure 1). The results revealed that in addition to phosphoserine and phosphothreonine, three of the four proteins contained phosphotyrosine. These observations lead us to conclude that Hrr25p is a dual specificity kinase.

Fission Yeast CKI Isoforms Are Dual-specificity Kinases

Like budding yeast, fission yeast has a four-gene family of CKI isoforms. Two of these genes, *hhp1+* and *hhp2+*, encode protein kinases that are homologous to Hrr25p and participate in DNA damage repair (Dhillon and Hoekstra, 1994). The other two genes, *cki1+* and *cki2+*, are more distantly related to Hrr25p and are found exclusively in the cytoplasm (Wang *et al.*, 1994b). To determine whether fission yeast CKI isoforms share dual specificity protein kinase activity, Hhp1, Hhp2, and Cki1 were expressed in *E. coli* and examined for the presence of phosphotyrosine.

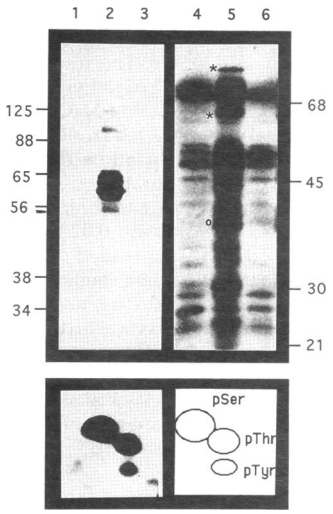

Figure 1. Hrr25p has dual-specific protein kinase activity. *E. coli* transformants were grown to mid-log phase and induced with IPTG. Lysates were prepared as previously described (Lindberg *et al.*, 1993). Lanes 1–3 are samples that were transferred to Immobilon and probed with a rabbit anti-phosphotyrosine antibody. The samples in lanes 4–6 are from in vivo–labeled phosphoproteins as shown in DeMaggio *et al.* (1992). Prestained molecular weight markers (Sigma) were used as size standards. The relative migration of Hrr25p is indicated by an arrow on the left hand side of lane 1. Lanes 1 and 4, vector alone; lanes 2 and 5, wild-type *HRR25*; lanes 3 and 6, catalytically inactive *hrr25-K38R*. The bottom left panel shows phosphoamino acid analysis of in vivo–labeled Hrr25p, and the right panel shows the migration pattern of ninhydrin-stained standards. Additional phosphoproteins that were examined for phosphoamino acids are indicated in lane 5. *, proteins that contain phosphoserine, phosphothreonine, and phosphotyrosine. O, protein that contains phosphoserine and phosphothreonine.

Studies on Hhp1 showed that, like Hrr25p, *E. coli* extracts containing wild-type Hhp1 reacted with anti-phosphotyrosine (Figure 2A). Phosphoamino acid analysis of Hhp1 confirmed the presence of phosphotyrosine (Figure 2B). Tyrosine kinase activity requires an active Hhp1 kinase as a mutation in the catalytic domain of Hhp1 that inactivates kinase activity (Hhp1-K40R) and destroys its protein-tyrosine kinase activity (Figure 2A). Recombinant forms of Hhp2 and Cki1 were also recognized by anti-phosphotyrosine antisera (see below). These results indicate that four different CKI isoforms from two distinct organisms can function as dual specificity protein kinases.

Tyrosine Autophosphorylation of CKI Isoforms In Vitro

To quantitate fission yeast CKI autophosphorylation activity, poly-His tagged Hhp1, Hhp2, and Cki1 were purified from *E. coli* and enzymologically characterized. The amount of protein-bound phosphate was estimated for each *S. pombe* isoform. Each isoform was highly

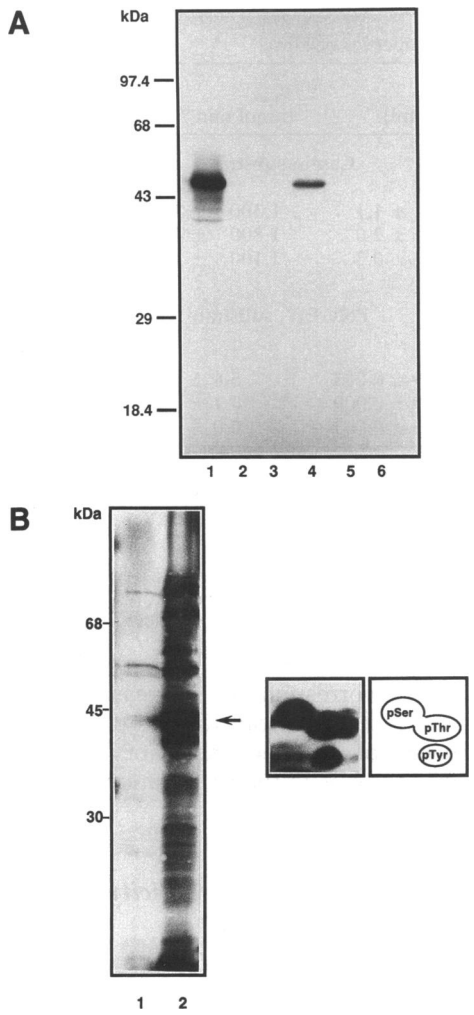

Figure 2. Hhp1 has dual-specific protein kinase activity. (A) Western blot analysis of lysates prepared from *E. coli* cells expressing either wild-type Hhp1 (lanes 1 and 4), Hhp1-K40R (lanes 2 and 5), or vector alone (lanes 3 and 6) that had been induced with IPTG (lanes 1–3) or from uninduced cells (lanes 4–6). Lysates were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-phosphotyrosine polyclonal antiserum. (B) *E. coli* cells carrying the vector alone (lane 1) or wild-type *hhp1+* (lane 2) were labeled with orthophosphate, and lysates prepared from these cultures were analyzed on a 10% SDS-polyacrylamide gel. The right panel shows the phosphoamino acid content of the 45-kDa phosphoprotein corresponding to autophosphorylated Hhp1.

phosphorylated, containing 4.8 ± 0.4 (Hhp1), 13.1 ± 1.2 (Hhp2), and 12.3 ± 0.6 (Cki1) mol phosphate/mol enzyme. The percentage of molecules containing phosphotyrosine was estimated by immunoprecipitation of each purified enzyme with antiphosphotyrosine antibody 4G10. The results revealed that 52.7 ± 0.6% of recombinant Hhp1, 36.9 ± 0.4% of Hhp2, and 23.9 ± 2.8% of Cki1 molecules contained phosphotyrosine as isolated from *E. coli*. We conclude that each of these three fission yeast CKI homologues contain significant

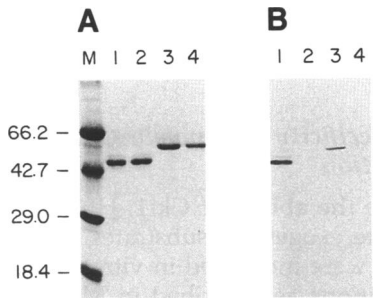

Figure 3. Dephosphorylation of Hhp1 and Hhp2 by PTPase. Purified Hhp1 and Hhp2 (1 μg each) were incubated (37°C for 30 min) in the presence or absence of 2 ng of PTPase as described in MATERIALS AND METHODS. Reactions were terminated by the addition of Na_3VO_4 (1 mM) then analyzed on 10% acrylamide gels. (A) Coomassie blue–stained gel containing Hhp1 and Hhp2 (0.5 μg samples). (B) Western analysis with antiphosphotyrosine antibody 4G10 on 0.25 μg samples. Lane 1, Hhp1 alone; lane 2, Hhp1 + phosphatase; lane 3, Hhp2 alone; lane 4, Hhp2 + phosphatase; lane M, molecular mass markers.

amounts of phosphotyrosine as a result of autophosphorylation.

To further characterize the autophosphorylation reaction, Hhp1 and Hhp2 were stripped of their tyrosine-bound phosphate by treatment in vitro with PTPase. As shown in Figure 3, this treatment quantitatively removed tyrosine phosphate from the proteins. Using the resultant dephosphorylated enzyme preparations, the rate of Hhp1 and Hhp2 autophosphorylation was assessed. As shown in Figure 4, the rate of Hhp1 tyrosine autophosphorylation was linear over a 30-min time course and was invariant over a 10-fold range of protein concentration. Similar results were obtained for Hhp2. On the basis of this behavior (Kuret and Schulman,

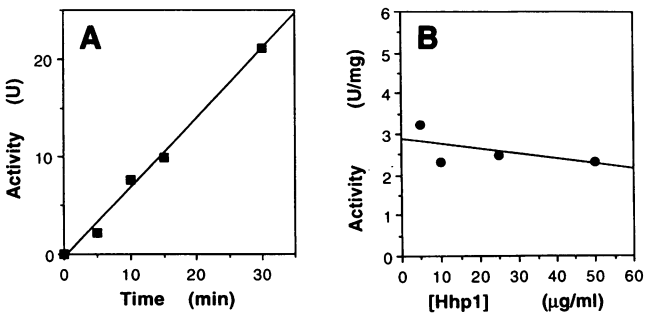

Figure 4. The effect of time and enzyme concentration on the tyrosine-specific autophosphorylation of Hhp1. Purified Hhp1 was dephosphorylated in vitro with T cell PTPase, subjected to autophosphorylation conditions, and analyzed for phosphotyrosine as described in MATERIALS AND METHODS. (A) The time course of tyrosine autophosphorylation is linear over 30 min when assayed at 25 μ/ml Hhp1. (B) The total tyrosine phosphate incorporated per μg of Hhp1 is constant over a 10-fold range of Hhp1 concentration (5–50 μg/ml), demonstrating that the mechanism of autophosphorylation is intramolecular.

1985), the mechanism of tyrosine autophosphorylation is intramolecular.

Substrate Specificity of S. pombe CKI Tyrosine Phosphorylation

To quantitate the ability of Cki1, Hhp1, and Hhp2 to phosphorylate exogenous substrates, purified enzyme preparations were incubated in vitro under phosphorylating conditions as described in MATERIALS AND METHODS. Exogenous substrates included casein, reduced carboxy methyl-lysozyme (RCM-L), and the synthetic peptide polymers poly-E_1Y_1, poly-E_4Y_1, poly-$E_1A_1Y_1$, and poly-$E_6A_3Y_1$. As expected, each of the three enzyme preparations efficiently phosphorylated casein. Hhp1 and Hhp2 phosphorylated the synthetic polymer poly-E_4Y_1 (Tables 1 and 2), but none of the other peptide polymers. Conversely, Cki1 could not phosphorylate any of the tyrosine-containing synthetic substrates, and phosphoamino acid analysis revealed that Cki1 also failed to phosphorylate tyrosine residues on casein or RCM-L. These results suggest that the Hhp gene products can phosphorylate both themselves and the tyrosine residues of exogenous substrates in vitro, whereas the tyrosine kinase activity of Cki1 is restricted to autophosphorylation.

To determine whether autophosphorylation affected the activity of CKI isoforms, Hhp1 and Hhp2 were dephosphorylated by Ser/Thr-specific and Tyr-specific phosphoprotein phosphatases in vitro and examined for their ability to phosphorylate exogenous substrates casein and poly-E_4Y_1. Results are summarized in Tables 1 and 2. Despite the large stoichiometry of phosphoserine and phosphothreonine, removal of this phosphate by treatment with phosphoprotein phosphatase-1γ (PrP-1γ) resulted in little or no change in the specific activity of Hhp1 or Hhp2, suggesting that autophosphorylation on Ser/Thr residues does not directly regulate CKI activity. Conversely, PTPase treatment resulted in a modest (two- to three-fold) but consistent increase in the Km for both casein and poly-E_4Y_1 phosphorylation and a corresponding decrease in catalytic efficiency of the enzymes (kcat/Km). Considering that the stoichiometry of tyrosine phosphorylation is less than 1 mol/mol enzyme in the preparations, the effects are significant.

Table 1. Kinetic properties of Hhp1

	Km (mg/ml)	V_{max} (nmol min^{-1} mg^{-1})	kcat/Km[a] (μM^{-1} min^{-1})
	Casein substrate		
Treatment			
None	0.77 ± 0.05	2,200 ± 100	2.8 ± 0.2
PTPase	1.8 ± 0.2	3,000 ± 200	1.6 ± 0.2
PrP-1γ	1.1 ± 0.2	1,900 ± 200	1.7 ± 0.4
	Poly-E_4Y_1 substrate		
Treatment			
None	0.015 ± 0.002	7.4 ± 0.2	0.66 ± 0.09
PTPase	0.028 ± 0.003	6.4 ± 0.2	0.31 ± 0.03
PrP-1γ	0.012 ± 0.001	6.1 ± 0.1	0.68 ± 0.06

[a] Calculated assuming the molecular masses of casein and poly-E_4Y_1 are 22 and 30 kDa, respectively.

Table 2. Kinetic properties of Hhp2

	Km (mg/ml)	V_{max} (nmol min^{-1} mg^{-1})	kcat/Km[a] (μM^{-1} min^{-1})
	Casein substrate		
Treatment			
None	3.9 ± 1.1	1,300 ± 300	0.35 ± 0.13
PTPase	7.2 ± 2.0	1,800 ± 400	0.26 ± 0.20
PrP-1γ	3.1 ± 0.3	1,100 ± 100	0.37 ± 0.05
	Poly-E_4Y_1 substrate		
Treatment			
None	0.029 ± 0.003	5.4 ± 0.2	0.27 ± 0.03
PTPase	0.089 ± 0.009	7.1 ± 0.4	0.12 ± 0.01
PrP-1γ	0.025 ± 0.001	10.0 ± 0.1	0.57 ± 0.02

[a] Calculated as in Table 1.

hhp1 from S. pombe Has Dual Specificity and Is Tyrosine Phosphorylated In Vivo

To determine if the properties of yeast CKIs in vitro were representative of the nonrecombinant forms of the enzymes and to determine if yeast CKI behaved as a dual-specificity protein kinase in vivo, an immune complex protein kinase assay similar to the that used to establish the dual-specific nature of Spk1p was employed (Zheng *et al.*, 1993). Hhp1 was examined in these experiments. Hhp1 was epitope-tagged at the C-terminus (Dhillon and Hoekstra, 1994) with a peptide derived from the influenza hemaglutinin protein (Tyers *et al.*, 1992). The resultant construct, in which *hhp1+* was expressed under a down-regulated *nmt1* promoter, was used to transform *S. pombe*. Transformants were grown to mid-log phase and lysed by mechanical disruption. Extracts were clarified by a low speed centrifugation. Hhp1 was immunoprecipitated from lysates with the monoclonal antibody 12CA5 and assayed for protein kinase activity. As shown in Figure 5, only immunoprecipitates containing wild-type Hhp1, and not Hhp1-K40R, incorporated [γ-^{32}P]ATP in an autophosphorylation reaction. To determine the phosphoamino acid specificity, radiolabeled bands were excised, subjected to partial acid hydrolysis, and examined by thin-layer two-dimensional electrophoresis. The results show that, like recombinant Hhp1 produced in bacteria, Hhp1 iso-

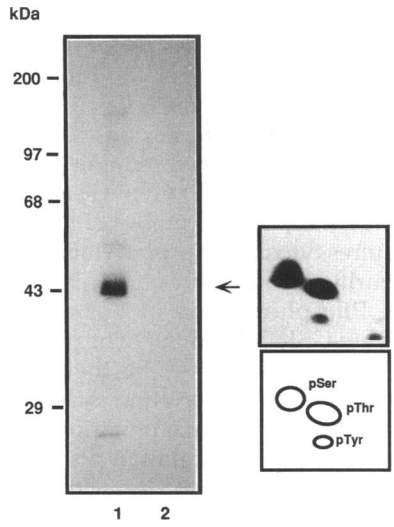

Figure 5. Hhp1 has an associated protein-tyrosine kinase activity. Immunoprecipitates of HA-Hhp1 (lane 1) or HA-Hhp1-K40R (lane 2) were used for protein kinase assays in vitro, and the reaction products analyzed by SDS-PAGE. The right panel shows a phosphoamino acid analysis of labeled Hhp1 and the corresponding ninhydrin-stained standards.

lated from fission yeast retains the ability to autophosphorylate on Tyr as well as Ser/Thr residues (Figure 5).

Wang *et al.* (1994b) have noted that cytoplasmic forms of fission yeast CKI associate with particulate fractions of the cell. During the course of these studies, it was observed that Hhp1 partitioned into two fractions, one fraction that was soluble in 1% Triton X-100 and a second low-speed pellet fraction that could be solubilized only by boiling with 1% SDS. After solubilization, the pellet-associated fraction could be immunoprecipitated with 12CA5 antibody. Subsequent Western analysis with the same antibody revealed that the pellet fraction was enriched for slower migrating forms of Hhp1 relative to the Triton X-100 soluble fraction (Figure 6). To examine the difference between these soluble and particulate forms, immunoprecipitates of each fraction were tested for their ability to react with anti-phosphotyrosine antibodies (Figure 6). The slower migrating forms of wild-type Hhp1 in both fractions cross-reacted with antiphosphotyrosine antibody (Figure 6, lanes 1 and 3), whereas the fastest migrating forms did not. Lysates containing the inactive Hhp1-K40R mutant did not show the slower migrating forms and Hhp1 was not detected in the pellet fraction.

The variation in levels of wild-type and Hhp1-K40R protein (Figure 6) was controlled by analyzing the expression of both forms of the protein kinase in a number of transformants. Several transformants containing varying levels of wild-type and Hhp1-K40R were analyzed, and each showed the same distribution. These studies revealed that both forms were generally equivalently expressed and, consistent with the data shown in Figure 6, lysates from all transformant cells expressing Hhp1-K40R lacked the slower migrating forms of the enzyme. These results suggest a possible role for tyrosine autophosphorylation of Hhp1 in vivo in the subcellular distribution of the enzyme.

DISCUSSION

Genes coding for CKI isoforms have been isolated from *S. cerevisiae* (Hoekstra *et al.*, 1991; Robinson *et al.*, 1992; Wang *et al.*, 1992), *S. pombe* (Dhillon and Hoekstra, 1994; Wang *et al.*, 1994b), mouse (Hoekstra, unpublished data), rat (Graves *et al.*, 1993), bovine (Rowles *et al.*, 1991), and human (Hoekstra, unpublished data) sources. The proteins encoded by these genes share, on average, >60% amino acid identity both within and between species (DeMaggio *et al.*, 1992). In relation to the entire protein kinase superfamily, CKI isoforms are more closely related to Ser/Thr protein kinases than to Tyr protein kinases. The results presented here reveal a novel feature for the enzyme subfamily, notably that four budding and fission yeast forms of CKI possess dual-specificity protein kinase activity.

In vitro phosphorylation by purified Hhp1 and Hhp2 isoforms of CKI indicate that phosphoacceptor proteins like casein are phosphorylated most efficiently by these protein kinases on Ser and Thr residues. Synthetic copolymer substrates for protein-tyrosine kinases are also phosphorylated by some of these enzymes, and removal of phosphate from the Tyr, but not the Ser/Thr residues, in Hhp1 and Hhp2 decreased the phosphotransferase activity of these protein kinases by two- to threefold. Moreover, Hrr25p, Hhp1, Hhp2, and Cki1 are autophosphorylated on tyrosine residues when expressed in *E. coli*, and Hhp1 is tyrosine phosphorylated in vivo.

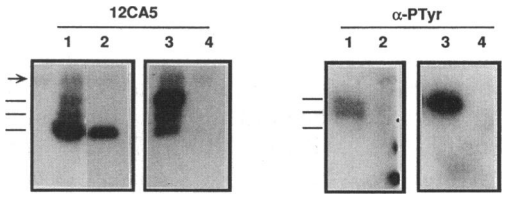

Figure 6. Hhp1 is tyrosine phosphorylated in vivo. Immunoprecipitates of the supernatant and pellet fractions of lysates from *S. pombe* cells carrying HA-Hhp1 or HA-Hhp1-K40R were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with either 12CA5 antibodies (left) or antiphosphotyrosine antiserum (right). Lanes 1 and 2 show immunoprecipitates from the supernatant fractions of Hhp1 and Hhp1-K40R lysates, whereas lanes 3 and 4 are immunoprecipitates from pellet fractions of Hhp1 and Hhp1-K40R. Bars indicate the differentially migrating forms of Hhp1, and the arrow denotes the position of the heavy chain of IgG. The approximately twofold lower expression of Hhp1-K40R (lane 2) compared to Hhp1 (lane 1) is transformant dependent. In parallel studies that examined several HA-tagged transformants, the level of Hhp1 and Hhp1-K40R varied between transformants, but lysates from all transformed cells expressing Hhp1-K40R lacked the slower migrating forms of the enzyme.

Hhp1 activity is required for this modification, suggesting that the tyrosine phosphorylation of Hhp1 in vivo is because of autophosphorylation. Thus, tyrosine phosphorylation of Hhp1 may have a physiological significance, and consistent with this suggestion we find differential fractionation profiles for the tyrosine-phosphorylated form.

In addition to demonstrating that yeast forms of CKI are dual-specificity protein kinases, tyrosine dephosphorylation of these enzymes affects their in vitro activity. Dephosphorylation of Hhp1 and Hhp2 with a serine/threonine phosphatase has little effect on the ability to phosphorylate casein or poly-E_4Y_1. Treatment of Hhp1 and Hhp2 with T cell PTPase quantitatively removes phosphotyrosine, and the dephosphorylated enzymes autophosphorylate on tyrosine in vitro. The tyrosine-dephosphorylated enzymes show an increased Km and reduced catalytic efficiency for casein and poly-E_4Y_1, suggesting that tyrosine phosphorylation of these enzymes might positively regulate their activity. Coupled with the observation that tyrosine-phosphorylated Hhp1 shows differential fractionation, these results suggest that the tyrosine kinase activity of these enzymes might be important for their optimum activity in vivo.

Hhp1, Hhp2, Cki1, and Hrr25p join a growing list of protein kinases in yeast that have been implicated as dual-specificity kinases. Based on their primary sequence, these protein kinases are structurally classified with the Ser/Thr protein kinase subfamily. The Mck1p protein kinase from baker's yeast was first identified by virtue of its ability to phosphorylate poly(E, Y) (Dailey *et al.*, 1990). The Mck1p gene product is important for yeast kinetochore function in mitosis and for early meiotic gene expression (Neigeborn and Mitchell, 1991; Shero and Hieter, 1991). The Spk1p protein kinase is an essential S-phase-specific gene product that is a nuclear Ser/Thr/Tyr kinase (Zheng *et al.*, 1993). It is suggested that Spk1p plays an important role in regulating DNA synthesis. In the mating type cascade in both yeasts, mitogen-activated protein kinases (MAPKs) and their activators have been identified. These protein kinases include Fus3p, Kss1p, and Ste7p in *S. cerevisiae* and Byr1 and Byr2 in *S. pombe* (Neiman *et al.*, 1993; Zhou *et al.*, 1993). Regulation of MAPK activity is via phosphorylation of a sequence motif, -TEY-, seven residues N-terminal to the conserved kinase domain VIII (-APE-), and phosphorylation of both Thr and Tyr is essential for MAPK signal transduction activity.

Of particular note for yeast dual-specificity protein kinases is that these enzymes are important for signal amplification. CKI isoforms are acidotrophic enzymes that are characterized by their ability to recognize prior phosphorylated substrates and consequently feed into second messenger pathways. MAPK isoforms are critical for the rapid amplification of external signals that stimulate transcription and proliferation. Given the diverse but common feature of signal amplification for these dual-specificity protein kinases, perhaps the capacity to phosphorylate Ser, Thr, and Tyr was independently acquired by a number of enzymes.

Hhp1 joins glycogen synthase kinase-3 (GSK-3), cdc2, and MAPK as protein serine/threonine kinases that are tyrosine phosphorylated in vivo (Featherstone and Russell, 1991; Davis, 1993; Hughes *et al.*, 1993). GSK-3 protein requires tyrosine phosphorylation for function, and, when purified from tissues, GSK-3 contains phosphotyrosine. Phosphorylation of Tyr on GSK-3β appears to activate GSK-3β and Ser/Thr phosphorylation inactivates (Wang *et al.*, 1994a). Further, the activity of GSK-3 for stimulating transcription is dependent upon tyrosine phosphorylation and a mutation in GSK-3 that abolishes tyrosine phosphorylation also alleviates the inhibition effect of GSK-3 on c-Jun activity (Hughes *et al.*, 1993). Hhp1 shows tyrosine phosphorylation in vivo, and this phosphorylation is required for Hhp1 subcellular distribution as assessed by cell fractionation (Figure 6). The tyrosine phosphorylation of Hhp1 appears to be autophosphorylation, and this suggests that Hhp1 might regulate its distribution within the cell through its ability to regulate its own phosphorylation state. Perhaps the biological function of CKI Tyr phosphorylation may not be direct regulation of enzyme activity but indirect regulation via control of cellular localization. Indeed, Anderson and coworkers (Brockman *et al.*, 1992) have shown that human CKI isoforms might participate in cell cycle progression as the CKIα isoform relocalizes from vesicular structures in G2 phase cells to the centrosome and mitotic spindles in mitotic cells.

One important question arising form the discovery that yeast forms of CKI can have associated protein-tyrosine kinase activity is whether human or other metazoan forms of this ubiquitous protein kinase show similar activity. To date, there is little experimental evidence regarding this notion. One report (Lu and Tao, 1986) has shown that an erythrocyte form of CKI is capable of protein-tyrosine kinase activity. In this study, CKI was purified to apparent homogeneity from a human erythrocyte cytosolic fraction. It was shown that the enzyme preparation could phosphorylate synthetic peptide substrates for tyrosine protein kinases like poly-EY, angiotensin II, and alkylated BSA on tyrosine. Like Hhp1 and Hhp2, the erythrocyte CKI preferred poly-E_4Y_1 over poly-E_1Y_1, and the erythrocyte enzyme was also capable of tyrosine phosphorylating band 3 protein and ankyrin. Clearly, one important challenge will be the elucidation of in vivo substrates for CKI.

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