Saccharomyces cerevisiae Yak1p protein kinase autophosphorylates on tyrosine residues and phosphorylates myelin basic protein on a C-terminal serine residue

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The serine/threonine protein kinase, Yak1p, functions as a negative regulator of the cell cycle in *Saccharomyces cerevisiae*, acting downstream of the cAMP-dependent protein kinase. In the present work we report that overexpression of haemagglutinin-tagged full-length Yak1p and an N-terminally truncated form (residues 148–807) lead to growth arrest in PKA compromised *yak1* null yeast cells. Both forms of recombinant Yak1p kinase were catalytically active and preferred myelin basic protein (MBP) as a substrate over several other proteins. Phosphopeptide analysis of bovine MBP by tandem MS revealed two major Yak1p phosphorylation sites, Thr-97 and Ser-164. Peptides containing each site were obtained and tested as Yak1p substrates. Both forms of Yak1p phosphorylated a peptide containing the Ser-164 residue with far more efficient kinetics than MBP. The maximal velocity ($V_{\max}$) values of the full-length Yak1p reaction were 110 ± 21 (Ser-164) and 8.7 ± 1.7 (MBP), and those of N-terminally truncated Yak1p were 560.7 ± 74.8 (Ser-164) and 34.4 ± 2.2 (MBP) pmol/min per mg of protein. Although neither form of Yak1p was able to phosphorylate

two generic protein tyrosine kinase substrates, both were phosphorylated on tyrosine residues *in vivo* and underwent tyrosine autophosphorylation when reacted with ATP *in vitro*. Tandem MS showed that Tyr-530 was phosphorylated both *in vivo* and *in vitro* after reaction with ATP. Pre-treatment with protein tyrosine phosphatase 1B removed all of Yak1p phosphotyrosine content and drastically reduced Yak1p activity against exogenous substrates, suggesting that the phosphotyrosine content of the enzyme is essential for its catalytic activity. Although the N-terminally truncated Yak1p was expressed at a lower level than the full-length protein, its catalytic activity and phosphotyrosine content were significantly higher than those of the full-length enzyme. Taken together, our results suggest that Yak1p is a dual specificity protein kinase which autophosphorylates on Tyr-530 and phosphorylates exogenous substrates on Ser/Thr residues.

Key words: dual-specificity protein kinases, protein phosphorylation, Yak1p.

INTRODUCTION

Functional modification of cellular proteins by reversible phosphorylation represents a major signalling mechanism in eukaryotic cells [1,2]. It is catalysed by a large family of protein kinases [3] that share structural similarities within a catalytic domain of about 300 amino acids encompassing 11 kinase subdomains [4]. Most protein kinases also contain regulatory domains on their N- and C-termini, which regulate the catalytic activity of the kinase, its sub-cellular localization and its interactions with cellular substrates and other proteins (see [4] for a review).

The family of protein kinases can be divided based on functional and structural parameters into three major subfamilies: (a) the protein tyrosine kinases phosphorylating the phenolic hydroxy group of tyrosine, (b) the protein Ser/Thr kinases phosphorylating the hydroxy group on the β -carbon of serine and threonine, and (c) the dual specificity protein kinases which catalyse phosphorylation of exogenous substrates and/or autophosphorylation on both tyrosine and serine/threonine residues [2,5–11]. The best characterized kinases of the dual-

specificity class are the mitogen-activated protein (MAP) kinase kinases (for example mammalian MEK1 and MKK4 and yeast Ste7p), which activate the MAP kinases, extracellular signal-regulated protein kinase (ERK)2, SAPK and Fus3p/Kss1p respectively by phosphorylating on both threonine and tyrosine residues [5,6,12,13]. Several dual-specificity kinases have been identified which autophosphorylate on tyrosine and serine/threonine residues but do not appear to phosphorylate exogenous substrates on tyrosine. These include transforming growth factor ('TGF') β receptor, and Mck1p and Hrr25p from *Saccharomyces cerevisiae* [14–16]. Recently, a new subfamily of dual specificity protein kinases which autophosphorylate on tyrosine and serine/threonine, but do not appear to phosphorylate exogenous substrates on tyrosine was identified. Members of this subfamily include mammalian DYRK1A/B/C, DYRK2 and DYRK3, *Drosophila* minibrain (mnbl), *S. cerevisiae* Yak1p, *Dictyostelium* YakA and, as yet, uncharacterized kinases from *Schizosaccharomyces pombe* (Z50142) and *Caenorhabditis elegans* (Z70308) [17–21]. The regions conserved amongst all members of this subfamily are restricted to the catalytic core. These features include an Ser-Ser-Cys motif following subdomain VII, and the con-

Abbreviations used: GST, glutathione S-transferase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; HA, haemagglutinin; mAb, monoclonal antibody; MBP, myelin basic protein; PKA, cAMP-dependent protein kinase; PTP, protein tyrosine phosphatase; Sc-His, minimal medium lacking histidine; PSD, post-source-decay; MALDI, matrix-assisted laser-desorption ionization; TOF, time-of-flight.

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served sequences HCDLKPEN and XYIQRFYR(S/A)PE in subdomains VI and VIII respectively [20]. Full-length DYRKs and *mnb*, as well as a fragment of the *YAK1* gene encoding the catalytic domain (amino acids 338–713), appear to autophosphorylate on tyrosine *in vitro* when expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* [20].

Yak1p functions as an antagonist of the Ras/cAMP-dependent protein kinase (PKA) pathway acting as a negative regulator of cell growth [17,22]. Recent data indicate that Yak1p functions downstream of PKA [23]. In the current model, active PKA down-regulates the activity of the transcriptional activators, Msn2p and Msn4p, leading to a decrease in expression of *YAK1* and other genes containing stress response element (STRE) sites [23]. Yak1p appears to be functionally similar to the *Dictyostelium* YakA, which is essential for starvation-induced development and growth arrest [21]. Overexpression of either Yak1p or YakA in a *cdc25* (temperature sensitive) yeast strain leads to G₁ arrest at the permissive temperature [21,22]. In addition, the sequence similarity between the two kinases extends beyond the catalytic core. Yak1p and YakA both contain poly-Gln and poly-Asn stretches, as well as conserved sequences immediately N-terminal to the kinase domain and between subdomains X and XI [21].

In contrast to Yak1p and YakA, *mnb* and DYRK1 may be acting as positive regulators of cell growth. In the absence of *mnb*, *Drosophila* lacks proper neuroblast cell formation and maintenance; furthermore, *DYRK1*, the closest known *mnb* homologue, has been mapped to the region of chromosome 21, which is triplicated in Down's syndrome, suggesting that *mnb* and DYRK1 function as positive mediators of cell growth [18,19]. However, Souza et al. [21] suggest that the loss of cell maintenance or formation, but rather the absence of a signal to arrest growth and induce differentiation of neuroblasts.

Although the Yak1p protein kinase was shown to phosphorylate casein and to exhibit cell-cycle related changes in activity and protein expression, little is known about its enzymic properties and precise biological role. In the present study we report the characterization of several unique enzymic features of Yak1p kinase. Our data indicate that Yak1p is a dual specificity protein kinase, which is phosphorylated *in vivo* and autophosphorylates *in vitro* on Tyr-530, but phosphorylates exogenous substrates on serine and threonine residues. We have also demonstrated that tyrosine phosphorylation is required for Yak1p catalytic activity.

EXPERIMENTAL

Materials

Anti-haemagglutinin (HA) monoclonal antibody (mAb), clone 12CA5, was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Detergents, electrophoresis reagents and protein determination reagents were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Bovine myelin basic protein (MBP) and Protein A-agarose were obtained from Gibco-BRL (Gaithersburg, MD, U.S.A.). Protein tyrosine phosphatase (PTP) 1B (catalogue number 14-109), and the anti-phosphotyrosine mAb, 4G10, were obtained from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). [γ -³²P]ATP and chemiluminescent reagent were obtained from Amersham Pharmacia Biotechnology Inc. (Piscataway, NJ, U.S.A.). The sources of peptides used in this study are shown in the Table captions. Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Construction of expression vectors

Oligonucleotide pairs, 5'-GCC-ACT-AGT-GCC-ATG-TAC-CCA-TAC-GAT-GTT-CCA-GAT-TAC-GCT-ATG-AAC-TCA-TCC-AAT-AAT-3' and 5'-GCC-ATC-GAT-TTA-TTC-TTC-GAC-AAT-GTG-3', and 5'-GCC-ACT-AGT-GCC-ATG-TAC-CCA-TAC-GAT-GTT-CCA-GAT-TAC-GCT-CCC-GCT-TAC-ACC-AGC-TCT-3' and 5'-GCC-ATC-GAT-TTA-TTC-TTC-GAC-AAT-GTG-3', were used to amplify full-length *YAK1* [17] and an N-terminal deletion (amino acids 148–807) respectively, using standard PCR conditions. In both cases, the 5' oligonucleotide contained a *SpeI* site and nucleotides encoding the HA epitope, and the 3' primer contained a *ClaI* site. Following T/A cloning and sequence confirmation of the cloned products, full length and N-terminally truncated *YAK1* were subcloned as a *SpeI*–*ClaI* fragment into p423GAL1, a high-copy yeast expression vector containing the *GAL1* promoter and *HIS3* as the selectable marker [24]. The resulting plasmids were termed p423GAL1-HA-YAK1 and p423GAL1-HA- Δ NYAK1, respectively.

Growth of yeast and preparation of cellular extracts

All media for growth were prepared as described previously, unless otherwise indicated [25]. Yeast strain SGY470 [*MATa ura3 leu2 trp1 his3 lys2 ade8 tpk1::URA3 tpk2-63(ts) tpk3::TRP1 yak1::ADE8*; a gift from S. Garrett, University of Medicine and Dentistry of New Jersey] was transformed by the lithium acetate procedure [26] with p423GAL1, p423GAL1-HA-YAK1, or p423GAL-HA- Δ NYAK1. Transformants were selected for growth on minimal medium lacking histidine (Sc-His). To perform spot assays, cells were grown in Sc-His overnight, cultures were normalized to similar D_{600} , serially diluted and spotted on to either Sc-His or Sc-His containing 2% galactose and 1% raffinose. Cultures for experiments were grown in liquid Sc-His to a D_{600} of at least 1.0, washed with Sc-His containing 2% galactose and 1% raffinose and resuspended in this medium to twice the original volume. The cells were then grown for 16–24 h at room temperature, washed once with water and, after centrifugation at 3200 g for 5 min at room temperature, the pellets were stored at –80 °C until use. To prepare lysates, cell pellets were thawed and resuspended at 1 ml/100 ml of original culture in lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl containing 10 μ g/ml each of aprotinin, leupeptin and tosyl-lysylchloromethane (Tos-Lys-CH₂Cl, 'TLCK'), 0.1 mM PMSF, 50 mM NaF, 0.2 mM Na₃VO₄ and 10 mM 2-glycero-phosphate]. Sterile acid-washed glass beads (0.5 ml) were added and cells were disrupted with ten 30 s intervals of vortex mixing. Nonidet P-40 was added to a final concentration of 2% and the mixtures were rocked at 4 °C for 30–50 min. Lysates were clarified by centrifugation at 20000 g for 10 min at 4 °C, and the supernatants were stored at –80 °C until use.

Immune-complex protein kinase assay

Equal amount of proteins (50 μ g, unless stated otherwise) from detergent extracts of yeast cells expressing full-length Yak1p, N-terminally truncated Yak1p or vector alone were immunoprecipitated by rocking overnight at 4 °C with 4 μ g of the anti-HA mAb and 100 μ l of a 20% suspension of Protein A-agarose in lysis buffer containing 1% Nonidet P-40. Under these conditions, maximal immunoprecipitation of both forms of the enzyme was achieved. Samples were then washed twice with lysis buffer, once with kinase assay buffer [25 mM Hepes, pH 7.5, 10 mM 2-glycerol phosphate, 0.2 mM Na₃VO₄] and suspended

in 20 μ l of kinase assay buffer containing (unless stated otherwise) 0.1 mM (3 μ Ci) of [γ - 32 P]ATP, 10 mM MgCl_2 and the protein or peptide substrate at the concentrations indicated in Table captions and Figure legends. After incubation for 10 min at 30 °C, the reactions were stopped by adding either 5 μ l of 5 $\times$ SDS sample buffer or 20 μ l of 0.15 M H_3PO_4 . The SDS-containing solutions were boiled and centrifuged at 20000 g for 5 min at room temperature. The supernatant proteins were resolved by SDS/PAGE on 14% polyacrylamide gels and the phosphorylated products were detected by autoradiography. Reactions stopped with H_3PO_4 were centrifuged and the phosphorylated products were isolated by spotting 20 μ l of the supernatant on to phosphocellulose (p81) filters. The filters were washed three times with 75 mM H_3PO_4 , three times with water and 32 P-labelling was monitored by scintillation spectroscopy. Kinase specific activity is expressed as pmol of P_i transferred from ATP to the phosphate acceptor/min per mg of protein present in the extract used for immunoprecipitation after subtraction of the blank values generated in the absence of substrate or in reactions using extracts from yeast transfected with empty vector. In either case, blanks were < 5% of total activity values. Protein concentration was determined using Bio-Rad reagent with BSA as a standard.

Mapping of substrate phosphorylation sites

MBP was phosphorylated by incubation with Yak1p and Mg^{2+} -ATP and digested with endoproteinase Lys-C for 2 h at 38 °C in 50 mM Tris/HCl, pH 8.8. An aliquot of the unfractionated protein digest was analysed by matrix-assisted laser-desorption/ionization-time-of-flight (MALDI-TOF) MS for the presence of phosphorylated peptides. Potential phosphopeptide precursor ions were selected for post-source-decay (PSD) analysis using a Bradbury-Nielsen ion gate. Phosphopeptides were isolated by reverse-phase HPLC using a 1-mm i.d. C_{18} column. Peptides, in solution A, were eluted with a 5–50% gradient of solution B (solution A: 2% acetonitrile containing 0.1% trifluoroacetic acid in water; solution B: 95% acetonitrile containing 0.1% trifluoroacetic acid in water) at 50 μ l/min. The column eluate was split after passing through the UV detector, so that 5 μ l/min was sent to a QTOF mass spectrometer and 45 μ l/min went to a fraction collector, where fractions were collected at 1 min intervals. Phosphopeptides were sequenced by nano-electrospray tandem MS (QTOF spectrometer).

Autophosphorylation and determination of phosphotyrosine content

To determine total autophosphorylation of Yak1p, washed immune complexes were suspended in 20 μ l kinase assay buffer containing 50 μ M (5 μ Ci) of [γ - 32 P]ATP and 10 mM MgCl_2 , and incubated for 10 min at 30 °C. Reactions were stopped by adding 5 μ l of 5 $\times$ SDS sample buffer, and the proteins were resolved by SDS/PAGE (10% polyacrylamide gels). Phosphorylated Yak1p was revealed by autoradiography. To determine phosphotyrosine content *in vivo* and tyrosine autophosphorylation of Yak1p, washed immune complexes were suspended in 20 μ l of kinase assay buffer with or without the addition of 50 μ M unlabelled ATP and 10 mM MgCl_2 . Samples without ATP were kept on ice, and those containing ATP were incubated for 10 min at 30 °C. All samples were resolved by SDS/PAGE (10% polyacrylamide), transferred to nitrocellulose, blocked in PBS/Tween-20 containing 5% (w/v) non-fat powdered milk for 1 h, and incubated for 1 h with anti-phosphotyrosine mAb (4G10). After three washes in PBS/Tween-20, membranes were incubated with horseradish-peroxidase-conjugated goat anti-

mouse IgG for 45 min and washed three times in PBS/Tween-20. The secondary antibody was detected by enhanced chemiluminescence.

To determine the sites of phosphorylation *in vivo* and *in vitro*, Yak1p immunoprecipitated from 1 mg of yeast protein was incubated without or with Mg^{2+} -ATP, purified by SDS/PAGE, as above, and stained with Coomassie Blue. Bands representing Yak1p were excised from the gel and digested with trypsin *in situ* as described previously [27]. The peptide mixture was desalted using a ZipTip (Millipore) according to the manufacturer's directions and enriched for phosphopeptides using immobilized metal-chelate affinity chromatography on a disposable micro-column [28]. The phosphopeptide mixture was again desalted on a ZipTip and an aliquot of the sample was analysed by MALDI-TOF to determine the molecular masses of the phosphorylated peptides. MALDI mass spectra [29] were recorded on a reflectron TOF mass spectrometer (Micromass TofSpec SE) equipped with a time-lag focusing source. Samples were prepared by mixing with an equal volume of matrix solution, which contained two internal mass standards at a concentration of 200 fmol/ μ l. A 0.5 μ l aliquot of the sample/matrix solution was applied to the MALDI target. The matrix solution was 10 μ g/ml α -cyano-4-hydroxycinnamic acid in ethanol/acetonitrile (50:50, v/v). Samples were then irradiated with 337 nm photons from a pulsed nitrogen laser (Laser Science) operating at 5 Hz. Phosphopeptides were sequenced by tandem MS [30] using a quadrupole TOF mass spectrometer (QTOF, Micromass) equipped with a nano-electrospray source [31].

RESULTS

Expression and activity of Yak1p in *S. cerevisiae*

YAK1 was engineered for expression in yeast under the control of the *GAL1* promoter as both an N-terminally HA-tagged full-length form and an N-terminally truncated form (amino acid residues 148–807) (Figure 1A). Overexpression of either protein in a strain compromised for PKA and lacking Yak1p (SGY470) caused growth arrest at 30 °C (Figure 1C) and slower growth at 23 °C (Figure 1B), indicating that both forms of the enzyme were functional. However, the expression level of the full-length protein was 2–3-fold higher than that of the N-terminally truncated (Δ N) form (Figure 2). The slow growth observed at 23 °C was not due to general toxicity caused by overexpression, since neither construct caused growth arrest in a wild-type strain at either temperature (results not shown).

Cells expressing the full-length or N-terminally truncated forms, or containing vector alone were lysed in a buffer containing 2% Nonidet P-40, and an anti-HA mAb immune-complex kinase assay was performed using bovine MBP as substrate. Both full-length and N-terminally truncated forms of the enzyme were able to catalyse phosphate transfer from ATP to MBP (Figure 2). MBP phosphorylation was compared with that of other protein substrates, each used at 10 μ g/reaction mixture. A much lower level of phosphorylation by either form of Yak1p was observed when casein was used as a substrate, and no phosphorylation of enolase, heat shock protein 27 (hsp27), histone or poly-Gly/Tyr was observed (results not shown). It was determined in preliminary experiments that the two forms of Yak1p were equally precipitated, since an anti-HA mAb immunoblot of the precipitated material indicated the ratio between the two forms was similar to that observed in the direct immunoblot shown in Figure 2. MBP phosphorylation, catalysed by either form of Yak1p, was better with Mg^{2+} than Mn^{2+} as the cation, was inhibited by 1 mM CaCl_2 , was linear with time up to 15 min and was similar when incubation was at 30 °C or 37 °C (results not

Figure 1 Overexpression of Yak1p complements a *yak1* deletion in a PKA compromised strain of *S. cerevisiae*

(A) Schematic diagram of full-length (FL) and N-terminally truncated (ΔN , amino acids 148–807) Yak1p. (B, C) Yeast strain SGY470 [*yak1* Δ , *tpk1* Δ , *tpk3* Δ , *tpk2(ts)*] was engineered to express two independent isolates of N-terminally HA-tagged Yak1p (full-length, rows 3 and 4; N-terminally truncated, rows 5 and 6) or vector alone (rows 1 and 2) under control of the *GAL1* promoter. Serial dilutions of normalized cultures were plated on to Sc-His (left) or Sc-His galactose/raffinose (Sc-His gal/raf) (right) and grown at either 23 °C (B) or 30 °C (C) for 7 days.

Figure 2 Protein expression of full-length and N-terminally truncated Yak1p and kinase activity with MBP

Yeast strain SGY470 was engineered to express either full-length Yak1p or an N-terminally truncated Yak1p (ΔN), and lysates were prepared as described in the Experimental section. (A) Total protein (30 μ g) was resolved by SDS/PAGE (10% polyacrylamide), transferred to nitrocellulose and immunoblotted with anti-HA mAb as described in the Experimental section. (B) Total protein (50 μ g) was immunoprecipitated with the anti-HA mAb and analysed for phosphorylation of bovine MBP (10 μ g) as described in the Experimental section. Reactions were analysed using SDS/PAGE (14% polyacrylamide) and phosphorylated bands were revealed by autoradiography (exposure for 20 min at -80 °C).

shown). Interestingly, although expressed at a lower level, the N-terminally truncated form of Yak1p was 3- to 5-fold more active against MBP than the full-length enzyme (Figure 2). The higher activity of N-terminally truncated enzyme against MBP was observed at all concentrations of MBP tested. Although the level of expression and kinase-specific activity varied between different yeast cultures, the enzymic properties and the activity/expression ratio of each form were similar in the different cultures.

Yak1p-catalysed phosphorylation sites in bovine MBP

HA-tagged Yak1p (50 μ g) was immunoprecipitated with the anti-HA mAb and incubated with 10 μ g of bovine MBP in the presence or absence of Mg^{2+} -ATP. MALDI-TOF analysis of 5% of the unfractionated endoproteinase Lys-C digests from the phosphorylated (with Mg^{2+} -ATP) and the non-phosphorylated (without Mg^{2+} -ATP) MBP revealed two apparent phosphate-containing peptides at m/z 1571.78 ($M^+ + H$) and 1682.80 ($M^+ + H$) in the phosphorylated sample (Figure 3A), corresponding to peptides of molecular mass 1570.77 and 1681.79, respectively. The 1570 Da species was consistent with the mass of a peptide containing residues 91–104 from MBP and one molecule of phosphate (calculated mass 1570.78 Da), whereas the 1681 Da peptide was consistent with the mass of the C-terminal Lys-C fragment from MBP incorporating residues 155–169 plus one molecule of phosphate (calculated mass 1681.78). Further support for the incorporation of phosphate into the 1570 and 1682 Da peptides is the presence of broad peaks (marked with an asterisk in Figure 3A) which arise by metastable loss of H_3PO_4 from the intact phosphopeptide molecular ion. These metastable peaks were not present in the spectrum of a non phosphorylated sample. However, to rule out any possibility that they arose by the loss of water from the phosphorylated peptide, we isolated the phosphopeptide molecular ions using an ion gate and performed MALDI-PSD analysis. Observation of the characteristic loss of H_3PO_4 (98 Da) from both peptides upon subsequent PSD analysis [27] confirmed that these peptides were, in fact, phosphorylated (results not shown).

The Lys-C digests were fractionated by reverse phase HPLC and the fractions were subjected to MS to provide an on-line read-out of the products. Fractions containing the two putative phosphopeptides were analysed by nano-electrospray MS, and the appropriate precursor ions were sequenced by collision-induced dissociation tandem MS. The spectrum obtained for the 1570.9 peptide (Figure 3B) is consistent with two phosphorylated forms of the sequence NIVTPRTPPPSQGK, which corresponds to residues 91–104 of bovine MBP. The major phosphorylation site was found to be Thr-97. The 181.04 Da mass difference between the b_6 and b_7 ions indicated the presence of phosphothreonine at this position. A very small amount of phosphate was also found at Thr-94. The evidence for phosphorylation at this second site was a low abundance series of $b_n-H_3PO_4$ ions starting at b_4 and ending at b_6 (results not shown). The evidence for phosphorylation at either site converges at the b_7 ion.

The tandem mass spectrum of the 1681 peptide (Figure 3C) is consistent with a monophosphorylated form of LGGRDSRS-GSPMARR, corresponding to the C-terminal residues 155–169 of bovine MBP. Although the tandem MS data clearly indicated that Ser-160 was not phosphorylated, it was not possible to distinguish between Ser-162 and Ser-164 as the sole site of phosphorylation in this peptide. However, results from a Yak1p kinase assay using synthetic peptides with Ser $\rightarrow$ Ala substitutions at position 164 or 162 as substrates supported the notion that Ser-164, not Ser-162, is the site of phosphorylation by Yak1p. As seen in Table 1, both forms of Yak1p phosphorylated the

Figure 3 Determination of Yak1p-induced phosphorylation sites on MBP

(A) Partial MALDI mass spectrum of an unfractionated Lys-C digest of MBP phosphorylated *in vitro*. Peptides modified by a single molecule of phosphate show an increase in mass of 80 Da. Peaks marked with an asterisk (*) arise from the metastable loss of H_3PO_4 from the corresponding phosphopeptide molecular ion. (B) Electrospray-chromatogram-ion-detection tandem mass spectrum of the $[M^{2+}]$ ion (m/z 786.4) from the 1570 Da phosphorylated peptide shown above. The mass difference between adjacent ions of the same series (i.e. b_n and b_{n+1}) corresponds to the in-chain mass of an amino acid residue. Ions marked b_n^{Δ} have the structure $b_n\text{H}_3\text{PO}_4$. (C) Electrospray-chromatogram-ion-detection tandem mass spectrum of the $[M^{3+}]$ ion (m/z 561.6) from the 1681 Da phosphorylated peptide shown above.

LGGRDSRAGSPMARR (Ser-162 → Ala) but not the LGGRDSRSGAPMARR (Ser-164 → Ala) peptide. Phosphorylation of Ser-164, which exists as an S/TP motif, is consistent with the Yak1p-induced phosphorylation of Thr-97, which also exists in a S/TP consensus sequence. Replacement of Pro-165 with alanine resulted in > 70% loss of activity of Yak1p (Table 1). In addition, replacing the S/TP consensus sequence of Ser-164 with an S/TP for Ser-162 did not allow Ser-162 to become an efficient phospho-acceptor, as Yak1p poorly phosphorylated the Gly-163 → Pro/Pro-165 → Ala peptide (Table 1).

Phosphorylation of bovine MBP on Thr-97 by MAP kinases has been reported previously [32,33]. However, we are not aware of any kinase that phosphorylates bovine MBP on Ser-164. Therefore the phosphorylation pattern of MBP by Yak1p appears to be unique.

Several other selected peptides were obtained from commercial sources and used as substrates in the Yak1p kinase assay alongside MBP and the Ser-164 peptide (LGGRDSRSGSPMARR). As shown in Table 2, the specific activity of either full-length or N-terminally truncated Yak1p with the Ser-164 peptide (1 mM) was about 9-fold higher than the specific activity with MBP (60 μM). A peptide (termed Thr-97 in Table 2), with the P RTP motif found in the NIVTPRTPPPSQGK tryptic fragment of MBP, was poorly phosphorylated by Yak1p (Table 2). Although the MS data showed that the NIVTPRTPPPSQGK tryptic fragment of MBP was phosphorylated mainly on Thr-97, with little phosphorylation of Thr-94, the low activity of Yak1p with the synthetic Thr-97 peptide (Table 2) may be due to sequence differences, particularly the lack of a residue corresponding to Thr-94 in MBP. Two additional peptides, GS1–10

Table 1 Phosphorylation of the Ser-164 peptide and its analogues by Yak1p

Anti-HA immune-complex Yak1p kinase assay of 50 μ g of protein from yeast expressing either full length or N-terminally truncated Yak1p was performed as described in the Experimental section. All peptides used in this study were from California Peptide Research Inc., Napa, CA, U.S.A. Protein substrates (1 mM) were incubated with the immunoprecipitated enzyme for 10 min at 30°C. Phosphorylated substrates were isolated on phosphocellulose filters. Kinase activity using the Ser-164 peptide as substrate was 894 ± 28 (full length) and 3447 ± 69 (N-terminally truncated form) pmol per mg of protein. The values are the percentage kinase activity compared with that of the Ser-164 peptide as substrate; means $\pm$ S.D. of triplicate determinations. Similar results were obtained in one additional experiment.

Peptide	Sequence	Yak1p activity	
		Full-length enzyme	N-terminally truncated form
Ser-164	LGGRDSRSGSPMARR	100 ± 3	100 ± 2
Ser-164 $\rightarrow$ Ala	LGGRDSRSGAPMARR	7 ± 2	4 ± 3
Ser-162 $\rightarrow$ Ala	LGGRDSRAGSPMARR	93 ± 5	100 ± 2
Pro-165 $\rightarrow$ Ala	LGGRDSRSGSAMARR	20 ± 2	28 ± 4
Ser-164 $\rightarrow$ Thr	LGGRDSRSGIPMARR	99 ± 7	81 ± 2
Gly-163 $\rightarrow$ Pro	LGGRDSRSPSPMARR	90 ± 2	95 ± 2
Gly-163 $\rightarrow$ Pro/Pro-165 $\rightarrow$ Ala	LGGRDSRSPSAMARR	25 ± 2	18 ± 1

Table 2 Phosphorylation of peptides by full-length and N-terminally truncated Yak1p

Experimental details are as described in Table 1. All peptides were used at 1 mM, MBP was 30 μ M. The Ser-164 peptide was purchased from California Peptide Research Inc., Napa, CA, U.S.A. Thr-669 peptide was from Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.; all other peptides were purchased from BACHEM Bioscience Inc., King of Prussia, PA, U.S.A. The Thr-97 peptide contains the 95–98 sequence (PRTP) of bovine MBP. In the peptide sequences shown, the N-terminus is on the left. Results are representative of two experiments with the N-terminally truncated) enzyme or three with the full-length form.

Peptide	Sequence	Yak1p activity (pmol/10 min per mg of protein)	
		Full-length enzyme	N-terminally truncated enzyme
Ser-164	LGGRDSRSGSPMARR	478	1809
Thr-97	APRTPGGRR	3	15
MBP4–14	EKRPSERSKYL	< 1	< 1
MBP68–82	YGSLPQKSQRSQDGN	< 1	< 1
GS1–10	PLSRTLVAACK	13	57
Thr-669	KRELVEPLTPSGEAPNQALLR	6	21
Tyr-418	RRLIEDNEYTARG	< 1	< 1
MBP		61	212

Table 3 Kinetic constants of the Yak1p kinase reaction

The anti-HA mAb immune-complex Yak1p kinase assay was performed as described in the Experimental section. Values for Michaelis–Menten kinetics (K_m and V_{max}) were calculated from double reciprocal plots of initial velocity of the enzyme versus substrate concentration, with at least nine different concentrations of a given substrate. The concentrations of ATP used were 1–1000 μ M, those of MBP were 1–150 μ M, and peptide concentrations were 25–2500 μ M. The incubation time (10 min) was within the linear range of the reaction, and concentrations of enzyme and substrates were chosen so that not more than 10% of the substrate would be converted. Results are the means $\pm$ S.E.M. of three to five independent determinations

Substrates	K_m (μ M)		V_{max} (pmol/min per mg of protein)	
	Full-length	N-terminally truncated	Full-length	N-terminally truncated
ATP with MBP	48 ± 7	36 ± 10		
ATP with Ser-164	72 ± 19	67 ± 7		
MBP	31 ± 8	34 ± 11	8.7 ± 1.7	34.4 ± 2.2
Ser-164	403 ± 56	393 ± 102	110.3 ± 21.1	560.7 ± 74.8
Thr-97	1213 ± 164	1166 ± 92	27.1 ± 0.9	13.2 ± 1.9

and Thr-669, were also poorly phosphorylated by Yak1p (Table 2). The GS1–10 peptide is derived from the first ten residues of glycogen synthase, where the Ser-7 residue is a site of protein kinase C phosphorylation, and is also phosphorylated by PKA [34,35]. The Thr-669 peptide is derived from the epidermal growth factor ('EGF') receptor cytoplasmic tail, with Thr-669 (in a S/TP motif) as the site phosphorylated by ERK2 and

p38 MAP kinases [33,36]. The specific activity of N-terminally truncated Yak1p towards each of the four peptide substrates was 4–5-fold higher than that of the full-length enzyme. Yak1p phosphorylated neither the two MBP-derived peptides (MBP4–14 and MBP68–82; Table 2) nor a tyrosine-containing peptide (Tyr-418, Table 2) derived from residues 412–422 of pp60^{c-src}, which is phosphorylated by tyrosine kinases [37].

Figure 4 Total [^{32}P]ATP and tyrosine autophosphorylation of Yak1p

(A) The indicated amount of protein from yeast cells expressing either full-length (FL) or N-terminally truncated (ΔN) Yak1p was immunoprecipitated, assayed for Yak1p kinase activity without exogenous substrate and resolved by SDS/PAGE. Phosphorylated bands were revealed by autoradiography (exposure for 45 min at -80°C). (B) Anti-phosphotyrosine mAb immunoblot of 150 μg of full-length (FL) and N-terminally truncated (ΔN) Yak1p before and after autophosphorylation. Immunoprecipitated samples were incubated in the absence or presence of Mg^{2+} ATP, resolved by SDS/PAGE and blotted with anti-phosphotyrosine mAb as described in the Experimental section.

Kinetic constants of the Yak1p kinase reaction

Since relatively high Yak1p specific activity towards the Ser-164 peptide was observed, we determined the kinetic constants of Yak1p for ATP, MBP, the Ser-164 peptide and the Thr-97 peptide using immune-complex kinase assays. The Michaelis

constant (K_m) and maximum velocity ($V_{\max}$) of the kinase reaction with each substrate were determined under conditions where the rate of phosphorylation was linear with respect to time and the concentration of the other substrate was not limiting. From double-reciprocal plots of initial velocity versus substrate concentration the apparent K_m and $V_{\max}$ with ATP, MBP, Ser-164 or Thr-97 as substrates were calculated. Values in Table 3 show that the Ser-164 peptide was a better substrate for both forms of Yak1p than MBP, with a 9-fold higher $V_{\max}$ of phosphorylation. Yak1p exhibited a single affinity for ATP regardless of the protein/peptide substrate used, giving apparent K_m [ATP] values around 40 μM . The apparent K_m value for phosphorylating any of the three phosphate acceptors was similar between full-length and N-terminally truncated forms of Yak1p, and both forms phosphorylated the Ser-164 peptide with an apparent K_m (approximately 0.4 mM) 3-fold lower and a $V_{\max}$ 50-fold higher than the corresponding values for the Thr-97 peptide (Table 3). The $V_{\max}$ value for Thr-97 phosphorylation was lower than that for MBP phosphorylation, and the apparent K_m value for this peptide was approximately 1.2 mM, which is comparable to its reported K_m value for the meiosis-activated MBP kinase (p44^{mpk}) from sea star oocytes [33,38]. Taken together, these results demonstrate that the truncated form of Yak1p exhibits higher catalytic activity than the full-length enzyme, and that both forms of the enzyme have far more efficient kinetics for Ser-164 phosphorylation than for MBP.

Phosphorylation *in vivo* and autophosphorylation *in vitro*

To determine if Yak1p autophosphorylates *in vitro*, equal amounts of yeast cell lysates were immunoprecipitated with anti-HA mAb and reacted with Mg^{2+} [^{32}P]ATP. Samples were then resolved on SDS/PAGE and incorporation of ^{32}P was revealed

Figure 5 Determination of autophosphorylation sites on Yak1p *in vitro*

(A) Partial MALDI mass spectrum of the Ga^{3+} -immobilized metal-affinity chromatography enriched fraction from a tryptic digest of Yak1p phosphorylated *in vitro*. (B) Electrospray-chromatogram-tandem mass spectrum of the $[M^{2+}]$ ion (m/z 605.8) from the 1210.6 Da phosphorylated peptide shown above.

by autoradiography. Both forms of Yak1p underwent autophosphorylation *in vitro*, which was significantly higher in the truncated protein (ΔN) than in the full-length enzyme (Figure 4A). To determine if Yak1p in *S. cerevisiae* was tyrosine phosphorylated *in vivo*, and if the observed autophosphorylation *in vitro* involved tyrosine residues, anti-HA mAb Yak1p immune complexes were either kept on ice or reacted with Mg^{2+} ATP to allow for autophosphorylation, resolved by SDS/PAGE and blotted with anti-phosphotyrosine mAb. Figure 4(B) shows that Yak1p was tyrosine phosphorylated *in vivo* and that the level of phosphotyrosine increased after incubation with Mg^{2+} ATP, indicating clearly that Yak1p autophosphorylates on tyrosine residues *in vitro*. It is also evident from the data shown in Figure 4 that the phosphotyrosine content *in vivo* and the level of tyrosine autophosphorylation *in vitro* were significantly higher in N-terminally truncated compared with full-length Yak1p. An ATP binding site mutant of Yak1p, in which the lysine residue at position 398 was replaced with alanine, was inactive against exogenous substrates and did not autophosphorylate *in vitro* (results not shown), indicating that the incorporation of phosphate into Yak1p (Figure 4) is most likely due to autophosphorylation of the kinase, not to other co-immunoprecipitating protein kinase activity.

To determine the *in vivo* and *in vitro* site(s) of phosphorylation, equal amounts of yeast cell lysates expressing full-length Yak1p were immunoprecipitated with anti-HA mAb, and either kept on ice or reacted with Mg^{2+} ATP to allow for autophosphorylation. The reaction mixtures were then resolved by SDS/PAGE and stained with Coomassie Blue. Yak1p appeared as a single band migrating at about 102 kDa, which was absent from samples from yeast cells transfected with vector alone (results not shown). The bands from both untreated and autophosphorylated proteins were excised and digested *in situ* with trypsin [27]. Phosphopeptides from the respective tryptic digests were isolated using microcolumn Ga^{3+} -ion immobilized metal-affinity chromatography ('IMAC') [28] and analysed by MALDI MS. Mass spectra from the autophosphorylated (Figure 5A) and the unphosphorylated (results not shown) samples showed an ion at $[M^+ + H]$ 1210.60, which corresponds to residues 526–534 of the Yak1p sequence with the addition of one molecule of phosphate (calculated $[M^+ + H]$ 1210.55). Figure 5(B) shows the tandem MS spectrum of the 1210 Da peptide from the autophosphorylated sample. A mass difference of 243 Da between the y_4 and y_5 ions indicated phosphotyrosine in the 5 position. Sequencing of the 1210 Da peptide from each sample by tandem MS showed unambiguously that the sequence 526–534 is phosphorylated *in vivo* and *in vitro* exclusively on Tyr-530, which is the second tyrosine residue in the YTY motif of Yak1p.

To determine if the phosphotyrosine content of Yak1p *in vivo* affects its activity, immunoprecipitated Yak1p was first reacted with PTP1B [39,40] to dephosphorylate the tyrosine residues and then assayed for substrate phosphorylation. To allow proper comparison of the treatments, Yak1p was treated with PTP1B in the presence (for untreated control) or absence (treated) of 0.5 mM Na_3VO_4 . In preliminary experiments it was determined that 0.5 mM Na_3VO_4 was sufficient to almost inactivate PTP1B. Whereas Yak1p treated with the phosphatase in the absence of vanadate lost most of its phosphotyrosine content, the presence of 0.5 mM vanadate provided complete protection from dephosphorylation, resulting in a phosphotyrosine content comparable with that observed in samples kept on ice (Figure 6B). Variability in the amount of immunoprecipitated material was minimal, as indicated by Western blotting with the anti-HA mAb (Figure 6A). As shown in Figure 6(C), removal of the phosphate groups from Yak1p tyrosine residues resulted in > 85% loss of

Figure 6 Effect of treatment of Yak1p with PTP1B on its phosphotyrosine content and substrate phosphorylation

Full-length (FL) and N-terminally truncated Yak1p (Δ) (100 μ g each, in triplicate) were immunoprecipitated (IP) with anti-HA mAb and washed as described in the Experimental section. One set was kept on ice (None), another set was incubated for 30 min at 30 °C in 20 μ l of Hepes buffer (pH 7.0) containing 1 μ g (activity of 2 nmol/min) GST-fusion full-length PTP1B (PTP), and the third set was incubated as above with PTP1B and 0.5 mM Na_3VO_4 (PTP + NaV). Samples were washed three times with kinase assay buffer containing 0.5 mM Na_3VO_4 , immunoblotted with anti-HA mAb (A) or anti-phosphotyrosine (Anti-pY) mAb (B) or assayed for kinase activity using 1 mM Ser-164 peptide (C), as described in the Experimental section. Results with full-length Yak1p only are shown in (A) and (B). Similar results were obtained in two independent experiments.

both full-length and N-terminally truncated kinase activity with the Ser-164 peptide, indicating that tyrosine phosphorylation of Yak1p is required for its catalytic activity against exogenous substrates. Similar reduction in kinase activity was observed when MBP was used as substrate (results not shown).

DISCUSSION

In the present study, we observed that expression of either full-length or an N-terminally truncated form of Yak1p kinase (residues 148–807) in a *S. cerevisiae* strain lacking Yak1p and compromised for PKA (*tpk1* Δ , *tpk2* Δ , *tpk3* (ts)) complemented the *yak1* deletion phenotype by causing cell arrest at 30 °C and slow growth at the permissive temperature (23 °C). This confirms earlier observations that Yak1p serves as negative regulator of the cell cycle in yeast [17,22]. Although a phenotypic difference between full-length and N-terminally truncated Yak1p was not observed in yeast, activity of the latter was 4–5 fold greater than the full-length enzyme, suggesting that an inhibitory element may be located within the first 147 amino acid residues of the protein. This region is rich in serine residues (24/147 residues)

and contains two putative PKA phosphorylation sites (residues 127 and 128; [17]). As Yak1p functions downstream of PKA [23] and was shown to be phosphorylated *in vitro* by PKA [22], it is tempting to speculate that phosphorylation of Yak1p on N-terminal serine residues (by PKA or another upstream kinase) serves to inhibit its activity. Loss of these sites in the truncated form would result in increased kinase activity as compared with the full-length enzyme.

Yak1p was previously reported to phosphorylate casein [22]. In the present study, we have shown that Yak1p prefers MBP as a substrate over casein and several other proteins, and that it is capable of phosphorylating the Ser-164 and Thr-97 residues of bovine MBP. Both forms of Yak1p phosphorylated a synthetic peptide (LGGRDSRSGSPMARR), corresponding to residues 155–169 of bovine MBP, with far more efficient kinetics than phosphorylation of native MBP. Although we were unable to distinguish between Ser-162 and Ser-164 as the sole site of phosphorylation using the tandem MS data, data generated using synthetic peptides with Ser → Ala substitutions demonstrated that Ser-164 is the phosphate acceptor for Yak1p. To our knowledge, no other kinase has been reported to phosphorylate the Ser-164 residue of bovine MBP.

Previously, it was shown that the catalytic portion of Yak1p alone autophosphorylates on tyrosine when expressed in *E. coli* as a GST fusion, however, catalytic activity was not demonstrated [20]. In the present work, we show that recombinant Yak1p kinase expressed in *S. cerevisiae* phosphorylates exogenous substrates on Ser/Thr residues, is tyrosine phosphorylated *in vivo* and undergoes tyrosine autophosphorylation *in vitro*, suggesting it is a dual specificity protein kinase. Yak1p contains a YTY motif in its activation loop (positions 528–530) [17], which is conserved in DYRK2 and DYRK3 [20], whereas a YQY motif is found in DYRK1 [19]. This is distinct from the TXY consensus sequence found in the activation loop of the Ser/Thr MAP kinases [12]. We have shown by MS-based microsequencing, that the second tyrosine (Tyr-530) in this motif was phosphorylated both *in vivo* and, after autophosphorylation, *in vitro*.

We were unable to demonstrate Yak1p-catalysed phosphorylation of either two generic tyrosine kinase substrates, enolase and poly-Gly/Tyr (results not shown), or a full-length peptide substrate (Table 2). In addition, anti-phosphotyrosine mAb immunoblots failed to detect tyrosine phosphorylation of histone (results not shown), which was weakly phosphorylated by Yak1p. Like Yak1p, four other members of this kinase family (DYRK1, DYRK2, DYRK3 and mnb) were reported to autophosphorylate on tyrosine, but from the limited reported phosphorylation studies, do not readily phosphorylate exogenous substrates on tyrosine [20]. Hence, Yak1p and members of this family may not be classical dual specificity protein kinases like GSK3, MEK1 and ESK [5,6,12,13], but rather evolutionary intermediates between Ser/Thr protein kinases and dual specificity protein kinases. Alternatively, the inability of Yak1p to phosphorylate exogenous substrates on tyrosine may simply be due to the lack of the appropriate recognition site within the selected substrates. The identification of Yak1p cellular substrates will supply direct evidence for the dual specific nature of the kinase.

Another important finding of this study is that tyrosine phosphorylation of Yak1p is required to maintain its catalytic activity towards exogenous substrates. Preincubation of Yak1p with PTP1B drastically reduced the phosphotyrosine content and the catalytic activity of the kinase when compared with samples incubated with inactive PTP1B (Figure 6). PTP1B had been successfully used to dephosphorylate tyrosine residues of various proteins *in vitro* to allow study of the function of the phosphotyrosine in these proteins [41–43]. Our observation that removal

of phosphate from tyrosine residues of Yak1p suppresses its catalytic activity further supports the unique dual-specific nature of this enzyme. In comparison, classic dual specificity protein kinases, such as the various isoforms of MEK, are activated by Ser/Thr (not tyrosine) phosphorylation catalysed by an upstream MEK kinase, thereby activating the p38 and ERK MAP kinases by dual threonine and tyrosine phosphorylation. The MAP kinases themselves are specific for Ser/Thr residues, but require phosphorylation on tyrosine and threonine residues for their activity. Conversely, Yak1p is a dual specificity protein kinase (autophosphorylates on tyrosine) in which the catalytic activity is highly dependent on its phosphotyrosine content, but phosphorylates exogenous substrates on Ser/Thr residues.

In summary, the present study demonstrates that the *S. cerevisiae* Yak1p is a unique dual specificity protein kinase which autophosphorylates on Tyr-530, requires phosphorylation on tyrosine for its catalytic activity and phosphorylates exogenous substrates on Ser/Thr residues.

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