Serine-threonine protein kinase activity of Elm1p, a regulator of morphologic differentiation in *Saccharomyces cerevisiae*

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Abstract **The *Saccharomyces cerevisiae* gene *ELM1* regulates morphologic differentiation and its nucleotide sequence predicts a novel protein kinase. Elm1p was expressed in yeast and insect cells and purified. Elm1p displayed protein kinase activity in autophosphorylation assays and towards exogenous substrates. Serine and threonine residues were identified as the acceptors in these reactions. These data together with previous genetic analysis of *ELM1* function indicate that phosphorylation on serine and/or threonine residues of a particular substrate or set of substrates by Elm1p is required for repression of the filamentous growth differentiation state.**

Key words: Serine-threonine protein kinase (*S. cerevisiae*); Pseudohyphal growth; Serine-threonine autophosphorylation; Baculovirus expression system; Kinase-negative mutant

1. Introduction

One of the developmental alternatives available to the ascomycete *Saccharomyces cerevisiae* is whether to assume the yeast form or the filamentous form during vegetative growth. In rich nutrient environments, *S. cerevisiae* normally adopts the yeast form, however, when specific environmental conditions are encountered cells adopt the filamentous form [1]. Filamentous form cells differ from yeast form cells in numerous characteristics, including cell shape, cell separation, invasive growth in agar medium, bud site selection and control of cell division cycle progression [1–3]. This cellular response in *S. cerevisiae* provides a convenient model system for investigating the molecular mechanisms controlling morphologic differentiation.

Three different known or putative protein kinases, as well as several transcription factors, have been implicated by genetic analyses in control of morphologic differentiation in *S. cerevisiae*. Little is known, however, about how these functions interact to regulate the switch between yeast form growth and the filamentous form. The MAP kinase cascade comprising the protein kinases Ste20p, Ste7p and Ste11p is essential for filamentous growth to occur in response to the environmental signal that normally stimulates this response [2,4]. The second kinase involved in this control process is cAMP-dependent protein kinase (PKA). Mutations in the ras protein RAS2, known to increase cAMP concentration by stimulation of adenylate cyclase activity, significantly stimulate filamentous growth [1,5], as do mutations that hyperactivate PKA directly [6]. Conversely, genetic manipulations resulting in a reduced cAMP level inhibited filamentous growth [7]. The transcription factors known to affect morphologic differentiation in *S. cerevisiae* are Phd1p [8], Sok2p [7] and Ste12p [4]. Ste12p is a downstream target of the MAP kinase pathway mentioned previously and Sok2p seemingly acts downstream of PKA [7].

A third putative protein kinase known to be involved in control of filamentous growth is Elm1p [9]. The protein product Elm1p is not closely related outside of the most highly conserved catalytic residues to any of the ≈400 known or putative protein kinase sequences and, thus, defines a novel branch of the kinase superfamily [10]. Deletion of *ELM1* caused multiple pseudohyphal growth characteristics regardless of the growth conditions, suggesting it functions as a repressor of filamentous growth in non-inducing conditions. Further understanding of how Elm1p acts to control the execution of a defined morphologic alteration that occurs in response to an external signal will require detailed characterization of its enzymatic activity and identification of the cellular target(s) of this unique protein. Towards that end, this study reports the expression and purification of fusion proteins that possess Elm1p catalytic activity and analysis of the enzymatic function of these molecules as serine-threonine protein kinases.

2. Materials and methods

2.1. Growth conditions for S. cerevisiae

Yeast strains were cultured at 30°C. Media used were YPD (1% yeast extract, 2% peptone, 2% glucose); SD (2% glucose, 0.7% yeast nitrogen base without amino acids, plus supplements as required at 20 μg/ml each); SG and SR (identical to SD except that 2% galactose or 2% raffinose, respectively, was present in place of glucose). Solid media contained 2% agar. *S. cerevisiae* was transformed using the lithium acetate procedure [11].

2.2. Antibodies

The 800 bp *Pst*I-*Eco*RI fragment of *ELM1* containing codons 94–362 was ligated in frame to the N-terminal coding region of the *E. coli trpE* gene in vector pATH21 [12], forming plasmid pCK20. *E. coli* transformants harboring pCK20 expressed a 59 kDa protein after induction of the *trp* promoter [12], matching the expected molecular weight of the fusion protein. This protein was recovered in the insoluble fraction of *E. coli*, partially purified by SDS-PAGE, and used to immunize rabbits according to standard procedures [13]. The resultant crude antiserum is termed anti-Elm1p. Commercially available antibodies used in this study were anti-GST (Molecular Probes, Eugene, OR) and anti-T7 (Novagen, Madison, WI).

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Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GST, glutathione S-transferase

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2.3. Construction of S. cerevisiae gene fusions and strains

The primary structures of the Elm1p proteins used in this study are represented in Fig. 1. The nucleotide sequence of *ELM1* is available as a segment of chromosome XI [14] (Genbank accession number X71621) running from the initiation codon at nt 4872 to the termination codon at nt 2950. Recombinant DNA manipulations were performed using standard procedures [13,15]. In all instances, the nucleotide sequence of the junctions between different segments of the fusion genes were determined.

Plasmid pCK35 contains the fusion gene *GAL:ELM1*, which expresses native Elm1p from the *GAL1* promoter. Plasmid pCK35 is based in the centromeric, *URA3*-marked vector pBM150 [16]. To construct pCK35, a *Bam*HI site was placed 7 nt upstream of the *ELM1* initiation codon by standard mutagenesis procedures. Another *Bam*HI site was placed ≈400 bp downstream of the *ELM1* termination codon by subcloning the genomic insert of plasmid pA1 [9] into a multiple cloning site vector. The 2.3 kb fragment bounded by these two *Bam*HI was cloned in the unique *Bam*HI site of pBM150, located downstream of the *GAL1* transcription initiation site, to form pCK35.

Plasmid pCK36 contains the fusion gene *GAL:GST-ELM1*, which expresses from the *GAL1* promoter a fusion protein termed GST-Elm1p that comprises *Schistosoma japonicum* GST and Elm1p; this plasmid is based in the *URA3*-marked vector pEG(KG) [17], which contains the 2 μm circle origin of replication. The *ELM1* coding region was provided essentially as described in the previous paragraph, except that the upstream *Bam*HI site was adjacent to the second *ELM1* codon in the correct reading frame for translational fusion to *GST*. The intermediate plasmid containing this form of the *ELM1* open reading frame is pCK37, which contains a unique *Sal*I site located ≈400 bp downstream of the *ELM1* termination codon in the multiple cloning sequence. The 2.3 kb *Bam*HI-*Sal*I fragment containing *ELM1* was ligated into the corresponding sites in the multiple cloning region of pEG(KG) to construct pCK36. Plasmid pEG(KG) provides the *CYC1* promoter region under control of the *GAL1-10* upstream activation sequence, followed by the *GST* coding sequence, followed by the multiple cloning region into which *ELM1* was inserted [17].

Plasmid pCK36-T2 contains the truncation allele *GAL:GST-ELM1T2*, which codes for the fusion protein GST-Elm1pT2. *GAL:GST-ELM1T2* was formed by digesting pCK36 with *Spe*I and *Hin*dIII, then recircularizing the plasmid fragment, thereby removing the region of *ELM1* downstream of codon 463 and eliminating 177 residues from the C-terminus. The 3′ non-coding sequences of *ELM1* were eliminated from the plasmid, so that transcription presumably terminates within the downstream vector sequence.

Plasmid pCK77 contains the fusion gene *GAL:GST-ELM1K117R*, which codes for the fusion protein GST-Elm1pK117R. This plasmid was formed essentially by replacing the 1180 bp *Pst*I-*Bgl*II fragment within the *ELM1* coding region in pCK36 with the equivalent fragment from YCpelm1 [9]. This fragment is identical to the wild-type sequence with the exception of a single base substitution that changes lysine codon 117 to an arginine codon.

2.4. Expression of Elm1p fusion proteins in S. cerevisiae and their purification

Yeast strain Y690 (*MAT***a**/*MAT*α *leu2/leu2 ura3/ura3 his3/his3* [pMA210]) was transformed to uracil prototrophy with pCK35, pEG(KG), pCK36 or pCK36-T2. Plasmid pMA210, present in strain Y690, is a *HIS3*-marked plasmid that expresses the transcriptional activator GAL4 from the *ADH1* promoter [18]. Cultures were grown to a density of 1×10^7 cells/ml in liquid selective SR medium; fusion protein expression then was induced by addition of galactose to 4% (v/v) and cells were harvested 8–12 h later. Total cell lysates were prepared from ≈1×10^9 cells broken by vortexing in the presence of glass beads and GST proteins were affinity-purified using glutathione-agarose beads, both according to standard procedures [13]. The beads were suspended in 50 μl of 1×Laemmli loading buffer for immunoblot analysis or suspended in 50 μl of 40 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 10 mM $MgCl_2$, 5 mM NaF for phosphorylation assays.

2.5. Purification of recombinant Elm1p from insect cells

Standard procedures were used for cell culture, baculovirus infection and isolation of viral genomic DNA [19,20]. The recombinant baculovirus transfer plasmid pCK45 was constructed by cloning the 2.3 kb *Bam*HI fragment of pCK37 (described above) into pBlueBacHisA (Invitrogen Corp., San Diego, CA). In this plasmid, codons specifying an N-terminal tag of six histidine residues and 15 amino acids from the N-terminus of bacteriophage T7 gene *10* were fused in frame to the N-terminal codon of *ELM1*. The T7 gene *10* fragment provides the epitope recognized by monoclonal antibody anti-T7. Recombinant baculovirus ELM1-XP resulted from co-transfection of Sf21 cells with 1 μg wild-type baculoviral DNA and 2 μg pCK45 and subsequent purification of recombinant baculovirus, as described [19]. The recombinant virus was purified from wild-type virus by three rounds of plaque purification. The fusion protein produced from ELM1-XP is termed HT7-Elm1p (Fig. 1).

Clarified cell lysates were prepared from insect cell cultures according to standard procedures [13]. HT7-Elm1p was partially purified by two successive column chromatography steps, first cation exchange chromatography using SP Sepharose (Pharmacia, Piscataway, NJ) and then affinity chromatography using Ni^{2+}-agarose (Qiagen, Chatsworth, CA). The ion exchange column was eluted with a continuous gradient of 0 to 1.3 M NaCl in 20 mM HEPES pH 7.5, 1 mM PMSF. The Ni^{2+}-agarose column was equilibrated in 20 mM $NaPO_4$ pH 7.5, 500 mM NaCl, 1 mM PMSF, then washed successively with (1) the same buffer at pH 6.3, (2) the pH 6.3 buffer containing 50 mM imidazole and (3) the pH 6.3 buffer containing 100 mM imidazole. HT7-Elm1p was eluted with the pH 6.3 buffer containing 500 mM imidazole. In both chromatography steps, the fusion protein was detected by immunoblot analysis and phosphorylation activity. Control fractions were from insect cells infected with wild-type baculovirus, isolated using the identical procedure.

2.6. Phosphorylation assays and phosphoamino-acid analysis

Protein fractions containing GST-Elm1p immobilized on glutathione agarose (20 μl of glutathione-agarose bead slurry, 100-500 ng protein) or HT7-Elm1p purified from insect cells (3 μl, 100–500 ng protein) and equivalent control fractions, were analyzed for protein kinase activity as follows. The 30 μl autophosphorylation reactions containing 40 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 10 mM $MgCl_2$, 5 mM NaF, 1 mM [γ-^{32}P]ATP (7,000 Ci/mmol) (ICN, Costa Mesa, CA) were incubated at 30°C for 30 min. Exogenous phosphoacceptors used in similar reactions were bovine histone H1 (4 mg/ml), bovine histone H2b (1 mg/ml), bovine casein (1 mg/ml) and bovine myelin basic protein (1.5 mg/ml) (Sigma Chemical Corp., St. Louis, MO). For phosphorylation assays, the reaction mixtures were analyzed directly by SDS-PAGE and autoradiography. For phosphoamino-acid analysis, the reactions were stopped by precipitation in 20% TCA in the presence of 20 μg/ml RNAse A and prepared for separation of amino acids on a Hunter thin-layer electrophoresis unit (CBS Scientific, Del Mar, CA) as described [21].

3. Results

3.1. Endogenous expression of ELM1

Towards the end of purifying Elm1p from *S. cerevisiae* cells, the endogenous levels of *ELM1* mRNA in asynchronous cultures were determined. *ELM1* mRNA was virtually undetectable by gel-blot hybridization analysis of total RNA from wild-type cells (Fig. 2A) although transcript was detected in polyadenylated RNA after several days exposure (data not shown). *ELM1* transcription was increased by directing transcription with the strong, inducible promoter of the *GAL1* gene [16]. The fusion gene *GAL:ELM1* was introduced into congenic *elm1* and *ELM1* strains as part of plasmid pCK35. RNA gel-blot hybridization analysis showed *ELM1* mRNA was strongly overexpressed specifically when cells were grown in galactose-containing medium (Fig. 2A). *GAL:ELM1* produced functional Elm1p, because the mutant morphologic phenotype of the *elm1* host strain was suppressed during growth in galactose, but not during growth in glucose (Fig. 2B) (carbon source alone had no effect on cell morphology). Polyclonal antibody anti-Elm1p failed to detect Elm1p in immunoblot analysis (Fig. 3, lane 1), despite the facts that high

levels of functional *ELM1* transcripts were present and that the antibody was able to detect an overexpressed Elm1p fusion protein in total soluble cell extracts (Fig. 3, lane 2). Taken together these data indicate Elm1p is a relatively non-abundant protein in asynchronous cell populations.

3.2. Purification of Elm1p fusion proteins from S. cerevisiae

Elm1p fusion proteins bearing a portion of GST were expressed in *S. cerevisiae* from the *GAL1* promoter. The gene fusion *GAL:GST-ELM1* functioned similarly to *GAL-ELM1* described above in that it expressed *ELM1* mRNA at high levels during growth in galactose medium and suppressed an *elm1* mutation in the host strain (data not shown). The fusion protein GST-Elm1p, therefore, provides Elm1p function in vivo.

In contrast to native Elm1p expressed from the *GAL1* promoter, GST-Elm1p accumulated in total soluble extracts to levels easily detectable by immunoblot analysis. Cells containing *GAL:GST-ELM1* on pCK36 produced a protein detected by anti-Elm1p of apparent molecular weight 100 kDa (Fig. 3, lane 2), which corresponds to the size of GST-Elm1p predicted from the nucleotide sequence of the fusion gene. This protein was present only in cells grown in the presence of galactose and it was detected in total soluble extracts also by anti-GST (data not shown). The 100 kDa protein was not present in total extracts of cells bearing pEG(KG), the empty vector that expresses unfused GST (data not shown). These data identify the 100 kDa protein as GST-Elm1p, which is responsible for the Elm1p function observed in vivo.

Accumulation of GST-Elm1p in cells afforded the ability to purify the protein by virtue of the affinity of GST for its substrate glutathione. Glutathione-agarose precipitates were analyzed by SDS-PAGE and immunoblot analysis. A 100 kDa protein was purified by this procedure that reacted

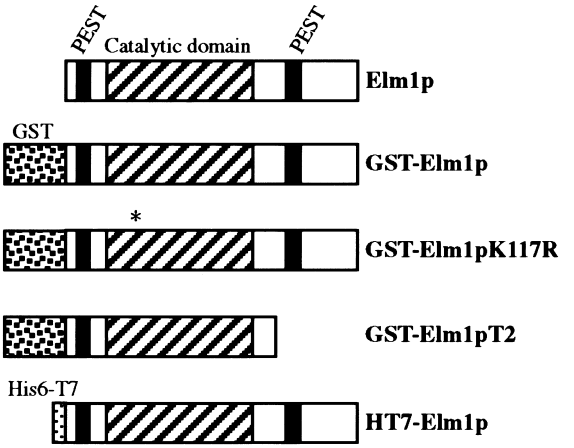

Fig. 1. Elm1p fusion proteins. The diagrams illustrate the primary structure of each protein, with the N-termini at the left of the figure. Proteins were expressed in *S. cerevisiae* under control of the *GAL1* promoter except for HT7-Elm1p, which was expressed in insect cells under the control of the baculovirus polyhedrin promoter. PEST regions were identified by the program PESTFIND [22]. The region designated as 'catalytic domain' is similar in amino-acid sequence to members of the protein kinase family. 'GST' indicates glutathione S-transferase protein. 'His6-T7' indicates six consecutive histidine residues followed by the T7 epitope tag. The asterisk indicates the position of the lysine to arginine substitution at residue 117. The figure is drawn to scale except for the His6-T7 block; full-length Elm1p comprises 640 amino acids.

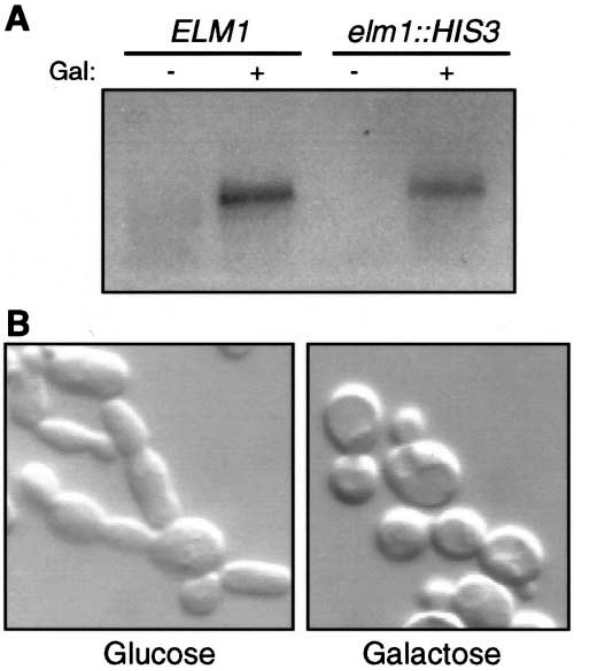

Fig. 2. Function of *GAL:ELM1*. (A) Steady-state levels of the *ELM1* transcript. Wild-type strain W303-1A (*MAT***a** *ura3 leu2 his3 ade2 trp1 ELM1*) [27] and the congenic *elm1::HIS3* mutant strain aWΔ*elm1H* [9], each containing *GAL:ELM1* as part of pCK35, were grown in selective raffinose medium to early log phase. The cultures were split into two portions; one of these was maintained in raffinose medium and the other was supplemented with galactose to a final concentration of 4%. *ELM1* transcripts were detected by RNA gel blot hybridization analysis, using as a probe the 1310 bp *Pst*I-*Eco*RI fragment from within *ELM1* [9]. (B) Complementation of *elm1::HIS3*. Strain aWΔ*elm1H* containing pCK35 was grown overnight on selective SD medium, then patched on either a fresh SD plate or a SG plate as indicated. After 24 h, cells were scraped from the plate, suspended in water and photographed using Nomarski optics.

with anti-Elm1p (Fig. 3, lane 4). As expected, this protein was also recognized by anti-GST (Fig. 3, lane 7) and was collected neither from cells bearing the control plasmid pEG(KG) (Fig. 3, lane 3), nor from cells grown in conditions that repress the *GAL1* promoter (Fig. 3, lane 6). As an additional control to verify the identity of the purified protein as GST-Elm1p, the C-terminal 177 codons of *ELM1* were deleted. *GAL:GST-ELM1T2* was able to restore wild-type yeast form morphology to *elm1* strains dependent on induction of the *GAL1* promoter (data not shown), indicating that the C-terminal 177 amino acids of Elm1p are not essential for function in vivo. Expression of *GAL:GST-ELM1T2* resulted in a protein detected by anti-Elm1p that was shortened relative to the full-length fusion protein (Fig. 3, lane 5). Together these data demonstrate that GST-Elm1p, a protein capable of providing Elm1p function in vivo, has been purified from *S. cerevisiae* cells.

3.3. Protein kinase activity of purified Elm1p fusion proteins

Protein kinase activity of GST-Elm1p was assayed by incubating glutathione-agarose precipitates with [γ-^{32}P]ATP in the presence or absence of various potential phosphoryl group acceptors. Activity was observed without addition of any exogenous acceptor molecule in precipitates from cells that accumulated GST-Elm1p (Fig. 4A, lane 2), but not from cells

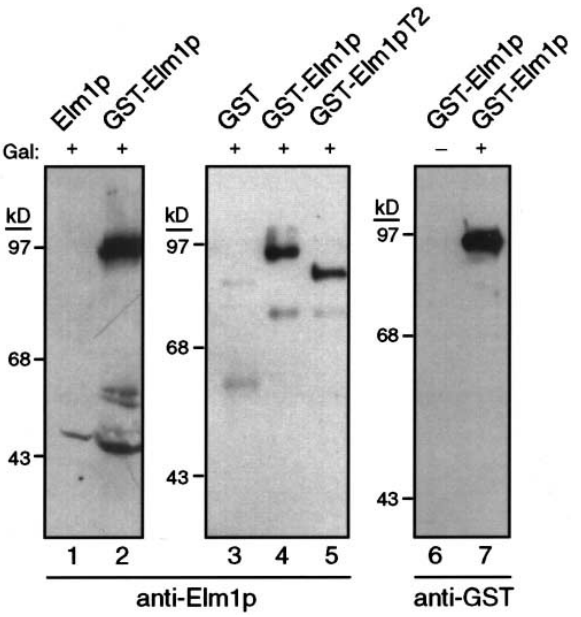

Fig. 3. Expression and purification of GST-Elm1p. Proteins from strain Y690 transformed with pCK35 (producing Elm1p), pCK36 (producing GST-Elm1p), pEG(KG) (producing GST) or pCK36-T2 (producing GST-Elm1pT2), as indicated, were detected by immunoblot analysis. The *GAL1* promoter was repressed by growth in raffinose (−) or induced by addition of galactose (+). The antibody used is indicated below each panel. The mobility in the same gels of standard proteins of known molecular weight is indicated. Lanes 1–2 contain total soluble cell extracts; lanes 3–7 contain glutathione-agarose precipitates.

expressing unfused GST (data not shown) or grown in repressing conditions for the *GAL1* promoter (Fig. 4A, lane 1). The major phosphoryl group acceptor in this reaction was ≈100 kDa, suggesting the reaction occurred by autophosphorylation of GST-Elm1p. This was verified by analysis of glutathione-agarose precipitates from cells containing *GAL:GST-ELM1T2*; these precipitates also displayed protein kinase activity and the acceptor was reduced in molecular weight in correspondence with the extent of the C-terminal deletion (Fig. 4A, lane 3). The glutathione-agarose precipitates from cells expressing GST-Elm1p also were capable of phosphorylating histone H1, casein and myelin basic protein (Fig. 4B). Again, no activity was present in precipitates from cells expressing unfused GST (data not shown) or from cells grown in repressing conditions for the *GAL1* promoter (Fig. 4B). The C-terminal deletion protein coded for by *GAL:GST-ELM1T2* also phosphorylated these three exogenous phosphoryl group acceptors (data not shown).

The following experiment ruled out the possibility that the observed kinase activity was due to a protein that co-fractionates with GST-Elm1p, as opposed to the fusion protein itself. GST-Elm1pK117R is a mutant protein identical to GST-Elm1p except for replacement of lysine residue 117 with an arginine. Based on known protein kinase structures, lysine 117 is expected to participate directly in catalysis such that the arginine substitution would eliminate Elm1p function without significantly affecting its structure. This mutation was known to inactivate the genetic function of *ELM1* [9] and, as expected, expression of the gene fusion *GAL:GST-ELM1K117R* failed to complement the cell morphology defect of an *elm1* mutant strain (data not shown). Immunoblot analysis indicated that GST-Elm1pK117R accumulated to the same level as did functional GST-Elm1p (data not shown). Glutathione-agarose precipitates from cell extracts expressing GST-Elm1pK117R were assayed for phosphorylation activity in parallel with GST-Elm1p precipitates. The mutant protein did not display activity either in the autophosphorylation reaction (Fig. 4A, lane 4) or towards histone H1 or myelin basic protein (data not shown). The protein kinase activity observed in GST-Elm1p precipitates, therefore, is due specifically to Elm1p.

3.4. Amino-acid specificity of Elm1p activity

The amino-acid specificity of phosphorylation by Elm1p was determined by phosphoamino-acid analysis. After autophosphorylation reactions using GST-Elm1p precipitates, the radioactive products were acid hydrolyzed and the resultant amino acids and phosphoamino acids were separated by two-dimensional thin-layer electrophoresis. Serine and threonine residues in Elm1p both were phosphorylated, but phosphotyrosine was not observed (Fig. 5C). Phosphorylated amino acids were not detected in the reaction products of glutathione-agarose precipitates from cells grown in repressing conditions for the *GAL1* promoter (Fig. 5A). Thus, Elm1p possesses serine-threonine protein kinase activity, as predicted from its amino-acid sequence similarity to previously characterized protein kinases [9,10].

3.5. Activity of Elm1p expressed in insect cells

To obtain Elm1p in nearly native form, as opposed to the GST-Elm1p fusion protein expressed in *S. cerevisiae*, *ELM1* was expressed in insect cells using the baculovirus system. The expressed protein, HT7-Elm1p, contains 21 amino acids fused

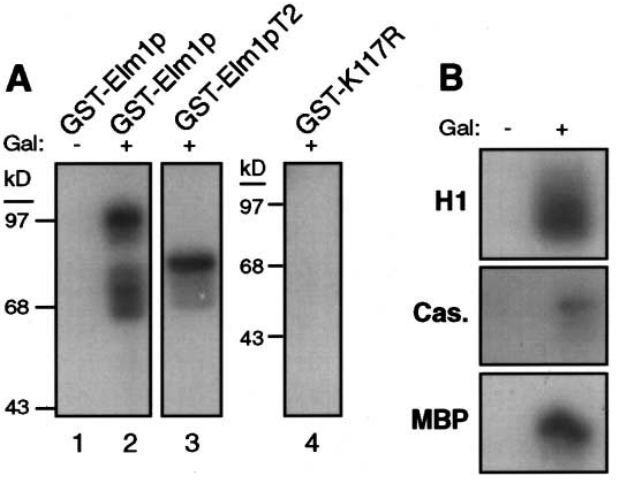

Fig. 4. Protein kinase activity of Elm1p fusion proteins. (A) Autophosphorylation. Proteins assayed were from strain Y690 transformed with pCK36 (producing GST-Elm1p), pCK36-T2 (producing GST-Elm1pT2) or pCK77 (producing GST-Elm1pK117R) as indicated. Glutathione-agarose precipitates were prepared from cells in which the *GAL1* promoter was repressed by growth in raffinose (−) or induced by addition of galactose (+). Precipitates were incubated with [γ-^{32}P]ATP, then analyzed by SDS-PAGE and autoradiography. The mobility in the same gel of protein standards of known molecular weight is indicated. (B) Phosphorylation of exogenous substrates. Glutathione-agarose precipitates from strain Y690 transformed with pCK36 (producing GST-Elm1p) were mixed with [γ-^{32}P]ATP and bovine histone H1, casein or myelin basic protein and analyzed as in panel A. Only the regions of the gels containing the exogenous phosphoacceptor protein are shown.

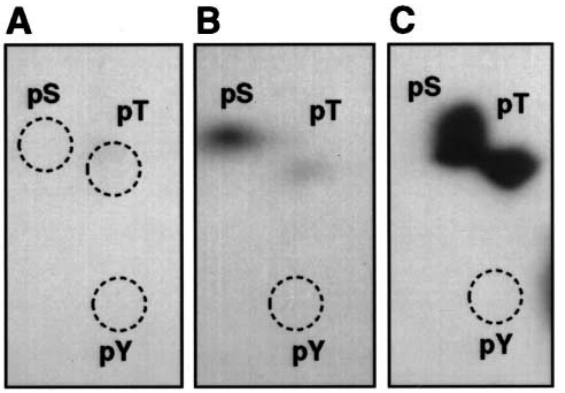

Fig. 5. Amino-acid specificity of Elm1p protein kinase activity. Phosphoamino-acid analysis was performed on the products of the autophosphorylation catalyzed by purified GST-Elm1p or HT7-Elm1p. The positions to which phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) migrate in the two-dimensional thin-layer electrophoresis separation were determined by staining of unlabeled standards. (A) Glutathione-agarose precipitate from *S. cerevisiae* strain Y690 containing pCK36 (producing GST-Elm1p), grown in raffinose medium to repress the *GAL1* promoter. (B) HT7-Elm1p partially purified from insect cells. (C) Glutathione-agarose precipitate from *S. cerevisiae* strain Y690 containing pCK36, grown in galactose-containing medium to induce the *GAL1* promoter.

to Elm1p at the N-terminus; the first six of these are histidine residues providing a Ni^{2+}-affinity tag and the next 15 provide an epitope tag recognized by monoclonal antibody anti-T7. Both anti-T7 and anti-Elm1p detected a protein of $\approx$72 kDa in soluble cell lysates, which was not detected in cells infected with wild-type baculovirus (data not shown).

HT7-Elm1p partially purified from Sf21 cell lysates exhibited enzymatic activity similar to GST-Elm1p. Phosphorylation of a 72 kDa protein was observed without addition of endogenous substrates to fractions from Ni^{2+}-affinity columns that contained HT7-Elm1p and this activity was absent from the control fractions (data not shown). Thus, the sole protein kinase activity present in the purified fractions is dependent on expression of HT7-Elm1p. Fractions containing HT7-Elm1p also phosphorylated histone H1, histone H2b and myelin basic protein (data not shown). Phosphoamino analysis revealed that phosphorylation occurred on serine and threonine residues (Fig. 5B), confirming the enzymatic activity of Elm1p as a serine-threonine protein kinase.

4. Discussion

The sequence of *ELM1* predicts a protein kinase that has no known close relative within the family [10]. Furthermore, the activity of Elm1p seemingly controls a developmental switch that regulates execution of a defined morphogenetic program. Thus, it is of interest to determine the biochemical activity of Elm1p and subsequently identify the proteins with which it interacts to accomplish control of morphologic development. The current study confirmed predictions made by its primary sequence that Elm1p is a protein kinase and determined that the targets of this enzyme, at least in vitro, are serine and threonine residue side-chains. Elm1p was found to be typical of protein kinases as they act in vitro, in that its substrate specificity is relatively broad.

The fact that Elm1p acts to phosphorylate other proteins implies that its target (or targets) stimulates filamentous growth when present in the non-phosphorylated state. Deletion of *ELM1* or a mutation that changes the catalytic lysine residue conserved in the protein kinase family to an arginine, causes filamentous growth in the absence of the normally required signaling regime [9]. Thus, lack of phosphorylation at a site that normally is modified by Elm1p results in filamentous growth. Conversely, phosphorylation of this site seemingly causes cells to maintain the yeast form. A possible mechanism of filamentous differentiation consistent with these biochemical data is that wild-type cells respond to the environment by initiating a signal transduction pathway that inactivates Elm1p.

Elm1p is likely to act at a point relatively far downstream in the signaling pathway leading to filamentous form growth. In this complex process, multiple signal inputs seemingly are needed to cause morphologic differentiation [1]. Elm1p's control of morphologic differentiation bypasses all of these diverse signals, arguing against the possibility that the protein kinase is involved in sensing a specific signal or that *elm1* mutations cause physiological conditions that mimic one or another of the signal components. Furthermore, *elm1* mutations seemingly cause all the multiple aspects of the filamentous form, implying the entire filamentous growth pathway is induced when Elm1p is absent, as opposed to the alternative that Elm1p is involved specifically in one aspect of the response, such as cell elongation. Thus, Elm1p is likely to function between the signaling cascade and the apparatus controlling the differentiation state. This hypothesis is consistent with the observation that the effects of *elm1* mutations on morphologic differentiation are independent of the function of *STE7*, *STE11* or *STE20* (C. Koehler, unpublished results). The protein kinases coded for these genes are part of a hierarchical MAP kinase cascade that is known to be required for morphologic differentiation of otherwise wild-type cells, presumably as an upstream component of the signaling mechanism [4].

HT7-Elm1p expressed in insect cells migrated during SDS-PAGE with an apparent molecular weight of 72 kDa. This size was $\approx$9 kDa larger than that predicted from the sequence of *ELM1* described previously [9]. Nucleotide sequence analysis of this chromosomal region as part of the yeast genome project [14], however, revealed a one base discrepancy from the initial published sequence [9]. The change in codon 539 of the initial predicted protein elongates the open reading frame by 77 codons, which causes the predicted molecular weight of HT7-Elm1p to correlate closely with the observed molecular weight. Thus, the predicted size of Elm1p must be revised to 640 amino acids (predicted molecular weight 71 987 Da), as opposed to the 563 residues predicted earlier [9]. The extension of the open reading frame is at the C-terminus beyond residue 539 and, thus, does not affect the predicted sequence of the protein kinase domain that ends at residue 400. The region of Elm1p beyond residue 463 was found to be dispensable for protein kinase activity in vitro and for genetic activity in vivo.

Elm1p seemingly is present at very low levels in cells. The low abundance of this protein can be explained in part by the fact that *ELM1* mRNA accumulates at very low levels in asynchronous populations. Overexpression of the *ELM1* coding sequence from the *GAL1* promoter resulted in accumulation of the protein product, but only if Elm1p was fused to GST at the N-terminus; native Elm1p expressed from the

same promoter did not accumulate to detectable levels, but did have genetic activity. One possible explanation for these observations is that Elm1p has a short half-life. Computational analysis revealed that Elm1p contains sequences termed PEST motifs; these are rich in proline, glutamate, serine and threonine residues and are proposed to serve as signals for rapid intracellular proteolysis [22,23]. The program PESTFIND [22] identified two potential PEST regions in Elm1p, one at the N-terminus in residues 24–50 and the other at the C-terminus in residues 487–515 (PESTFIND scores 10.6 and 1.8, respectively). In addition to these algorithm-defined regions, Elm1p is generally rich in P, E, S and T residues in the C-terminal extension downstream of the protein kinase domain (37% P, E, S, T residues in 200 total residues). Other *S. cerevisiae* proteins with PEST motifs are the G1 cyclin Cln3 [24,25] and the transcriptional regulator Gcn4 [26]; both of these have short half-lives mediated by the PEST sequences. The abundance of Elm1p may be regulated in a similar fashion. Addition of GST at the N-terminus may interfere with recognition of the N-terminal PEST sequence and, thus, stabilize the protein, explaining why GST-Elm1p expressed from the *GAL1* promoter accumulates whereas native Elm1p expressed from the same promoter does not.

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