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Aberrant Protein Phosphorylation at Tyrosine Is Responsible for the Growth-inhibitory Action of pp60$^{v\text{-}src}$ Expressed in the Yeast *Saccharomyces cerevisiae*

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Expression of pp60$^{v\text{-}src}$, the transforming protein of Rous sarcoma virus, arrests the growth of the yeast *Saccharomyces cerevisiae*. To determine the basis of this growth arrest, yeast strains were constructed that expressed either wild-type v-*src* or various mutant v-*src* genes under the control of the galactose-inducible, glucose repressible *GAL1* promoter. When shifted to galactose medium, cells expressing wild-type v-*src* ceased growth immediately and lost viability, whereas cells expressing a catalytically inactive mutant (K295M) continued to grow normally, indicating that the kinase activity of pp60$^{v\text{-}src}$ is required for its growth inhibitory effect. Mutants of v-*src* altered in the SH2/SH3 domain (XD4, XD6, SPX1, and SHX13) and a mutant lacking a functional N-terminal myristoylation signal (MM4) caused only a partial inhibition of growth, indicating that complete growth inhibition requires either targeting of the active kinase or binding of the kinase to phosphorylated substrates, or both. Cells arrested by v-*src* expression displayed aberrant microtubule structures, alterations in DNA content and elevated p34^{CDC28} kinase activity. Immunoblotting with anti-phosphotyrosine antibody showed that many yeast proteins, including the p34^{CDC28} kinase, became phosphorylated at tyrosine in cells expressing v-*src*. Both the growth inhibition and the tyrosine-specific protein phosphorylation observed following v-*src* expression were reversed by co-expression of a mammalian phosphotyrosine-specific phosphoprotein phosphatase (PTP1B). However a v-*src* mutant with a small insertion in the catalytic domain (SRX5) had the same lethal effect as wild-type v-*src*, yet induced only very low levels of protein-tyrosine phosphorylation. These results indicate that inappropriate phosphorylation at tyrosine is the primary cause of the lethal effect of pp60$^{v\text{-}src}$ expression but suggest that only a limited subset of the phosphorylated proteins are involved in this effect.

INTRODUCTION

In animal cells, protein-tyrosine phosphorylation is a key regulator of cell proliferation, morphology and differentiation. Three classes of protein kinase are known that phosphorylate tyrosine residues. The receptor tyrosine kinases, such as the epidermal growth factor receptor, undergo autophosphorylation at tyrosine upon binding ligand, creating a site to which signaling molecules can bind (Pazin and Williams, 1992). The non-receptor tyrosine kinases, of which the prototype is the pp60^{src} kinase encoded by the v-*src* proto-oncogene, are believed to act as effectors of transmembrane receptors that lack a cytoplasmic catalytic domain (Bolen *et al.*, 1992; Cooper and Howell, 1993). The third class includes the dual-specificity kinases (Lindberg *et al.*, 1992), which phosphorylate at both tyrosine and serine (or threonine) residues. Phosphotyrosyl residues are dephosphorylated by phosphotyrosine-specific or by dual-specificity phosphatases (Tonks *et al.*, 1989). The level of tyrosine phosphorylation of cellular proteins reflects the relative activities of the kinases and phosphatases that act upon them.

In budding and fission yeasts, which have been extensively used in studies on growth regulation and cell cycle control, tyrosine-specific kinases of the receptor or nonreceptor type have not yet been identified. However yeasts do contain a number of dual-specificity kinases. For example, in response to pheromone, the *Saccharomyces cerevisiae FUS3* and *KSS1* protein kinases, which are related to mammalian MAP (mitogen activated protein) kinases, are phosphorylated at threonine and tyrosine by the *STE7* kinase, which is itself homologous to MAP kinase kinase (Gartner *et al.*, 1992; Errede *et al.*, 1993) (Ma, Cook, and Thorner, unpublished observations). In *Schizosaccharomyces pombe*, the activity of the $p34^{cdc2}$ kinase is regulated by the $wee1^+$ and $mik1^+$ kinases; the $wee1^+$ kinase is a dual-specificity kinase which phosphorylates Tyr15 of $p34^{cdc2}$ but itself undergoes autophosphorylation at tyrosine and serine (Featherstone and Russell, 1991; McGowan and Russell, 1993). The homologous $p34^{CDC28}$ of *S. cerevisiae* is also phosphorylated at Tyr19, but the function of this phosphorylation is unclear (Amon *et al.*, 1992; Sorger and Murray, 1992). Other dual-specificity kinases identified in budding yeast include the Mck1 kinase, which undergoes autophosphorylation at tyrosine and serine but phosphorylates exogenous substrates at serine and threonine (Lim *et al.*, 1993), and Spk1 (Stern *et al.*, 1991; Zheng *et al.*, 1993), which phosphorylates proteins at serine, threonine, and tyrosine. Both budding and fission yeasts also contain phosphotyrosine-specific and dual-specificity phosphoprotein phosphatases. In *S. pombe* the $cdc25^+$, $pyp1^+$, $pyp2^+$ and $pyp3^+$ phosphatases regulate the activity of $cdc2^+$ (Kumagai and Dunphy, 1991; Millar *et al.*, 1992; Ottilie *et al.*, 1992). In *S. cerevisiae* several phosphotyrosine-specific or dual-specificity phosphoprotein phosphatases have been identified, including the *PTP1, PTP2, PTP3, CDC14, yVh1,* and *MIH* gene products, but their functions are as yet undetermined (Russell *et al.*, 1989; Guan *et al.*, 1991; Guan *et al.*, 1992a; Guan *et al.*, 1992b; James *et al.*, 1992; Wan *et al.*, 1992). The presence of both dual-specificity protein kinases and phosphotyrosine phosphatases in budding yeast indicates that tyrosine phosphorylation is involved in the regulation of yeast cell growth, metabolism, or proliferation.

One approach for exploring the function of vertebrate regulatory or signaling proteins is to examine their phenotypic effects when expressed in a heterologous organism, such as yeast. Characterization of the phenotypic changes induced by vertebrate proteins have often provided insights into regulatory processes that are conserved between yeast and vertebrates. Moreover the identification of mutations in the vertebrate protein that affect its ability to produce phenotypic effects in yeast provides means for analyzing its structure and function. Some time ago, two different groups independently introduced the tyrosine-specific protein kinase encoded by the v-*src* oncogene into *S. cerevisiae*, and observed that expression of $pp60^{v\text{-}src}$ leads to growth arrest (Brugge *et al.*, 1987; Kornbluth *et al.*, 1987), but the mechanism of the *src*-induced growth arrest was not explored.

The retroviral transforming protein $pp60^{v\text{-}src}$ is an activated derivative of the cellular $pp60^{c\text{-}src}$ tyrosine kinase, which has an as yet unknown function in both dividing and post-mitotic cells (Parsons and Weber, 1989). The N-terminus of both $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ is myristoylated, and myristoylation of these proteins is necessary for their membrane attachment and the ability of $pp60^{v\text{-}src}$ to induce cell transformation. The C-terminal segment of $pp60^{src}$ contains the catalytic (kinase) domain. The N-terminal half of $pp60^{src}$ contains the SH2 and SH3 (SH for "*src* homology") domains, which are noncatalytic segments conserved between *src*-related proteins and an ever growing number of other molecules that participate in cellular signal transduction pathways (Pawson and Gish, 1992). SH2 domains mediate protein-protein interactions by binding phosphotyrosyl residues within particular sequence contexts and thereby permit the assembly of complexes containing tyrosine phosphorylated receptors and signaling molecules or small adapter proteins (Pawson and Schlessinger, 1993). Mutations in the SH2 domain of $pp60^{v\text{-}src}$ or activated $pp60^{c\text{-}src}$ can render these proteins partially or completely defective for transformation of certain cell types (DeClue and Martin, 1989; Hirai and Varmus, 1990). Such SH2 mutations affect the kinase activity and intracellular localization of $pp60^{src}$ and as a result alter the pattern of the phosphorylation of cellular substrates (DeClue and Martin, 1989; Hirai and Varmus, 1990; Brott *et al.*, 1991; Fukui *et al.*, 1991; Liebl *et al.*, 1992). SH3 domains bind to specific proline-rich sequences in other signaling molecules, notably guanine-nucleotide exchange proteins that regulate the function of small GTP-binding proteins (Obar *et al.*, 1990; Ren *et al.*, 1993). Although the precise role of the SH3 domain in c-*src* function is unknown, mutations in this segment of $pp60^{v\text{-}src}$ affect the association of $pp60^{src}$ with certain cellular proteins and the resulting morphology of the transformed cell (Pawson and Gish, 1992). To date, no SH2-containing proteins have been definitively identified in yeast, although the nuclear protein Spt6 contains a region that displays sequence similarity to authentic SH2 domains (Maclennan and Shaw, 1993). SH3-like domains have been identified in yeast proteins involved in cytoskeletal function and budding (Wasenius *et al.*, 1989; Drubin *et al.*, 1990; Rodaway *et al.*, 1990; Chenevert *et al.*, 1992).

To determine the mechanism by which v-*src* arrests the growth of *S. cerevisiae*, we have examined the effects of expression of both wild-type v-*src* and a variety of mutant forms of this gene on cell growth, viability, morphology and DNA content. We also examined the pattern of protein phosphorylation at tyrosine in cells expressing wild-type v-*src* or v-*src* mutants, and

whether the effects of v-*src* could be reversed by expression of a known phosphotyrosine-specific phosphatase. While this manuscript was in preparation, an analysis of the effects of v-*src* expression in *S. cerevisiae* was reported by another group (Boschelli, 1993; Boschelli *et al.*, 1993).

MATERIALS AND METHODS

Plasmid and Strain Constructions

Yeast strains containing multicopy plasmids expressing wild-type or mutant v-*src* genes from the *GAL1* promoter were constructed as follows. Plasmids carrying either the wild-type or various mutant v-*src* genes in the pIRL19 vector (DeClue and Martin, 1989) were linearized by digestion with *Nco*I, which cleaves at the initiation codon of *src*. The termini of the linearized plasmids were converted to flush ends by incubation with the Klenow fragment of *Escherichia coli* DNA polymerase I and ligated with phosphorylated SalI linkers. The plasmids were then digested with *Sal* I and *Hind*III to release the v-*src* inserts, and the resulting *Sal* I-*Hind*III v-*src* fragments were then ligated into *Sal* I- and *Hind*III-digested yeast 2μm-DNA plasmid YEp51, which contains the *GAL1* promoter and the *LEU2* marker (Broach *et al.*, 1983). Yeast strain YPH499 (*MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*), the otherwise isogenic MATα strain YPH500, or the diploid strain YPH501 (Sikorski and Hieter, 1989) were transformed by electroporation and Leu$^+$ transformants were selected on complete minimal medium lacking leucine.

Yeast strains containing one or two integrated copies of galactose-inducible v-*src* were constructed as follows. An integrating plasmid containing the LEU2 marker, YIp51*src*, was derived from YEp51*src* by removing the *Xba* I-*Aat*II fragment, which contains the 2μ DNA origin of replication. To generate an integrating plasmid containing the *URA3* marker, a *Sma* I-*Hind*III fragment from YEp51*src* containing the *GAL1* promoter and the v-*src* gene was subcloned into the SmaI and HindIII sites of the vector YEp352; the 2μm origin sequence was excised from the resulting plasmid by digestion with *Nar* I and *Hpa* I and religation, generating plasmid YIp352*src*. Plasmid YIp51*src* was linearized by digestion with *Eco*RI, which cleaves within the *LEU2* gene, and YIp352*src* was linearized by digestion with *Eco*RV, which cleaves within the *URA3* gene. Strain YPH500 was transformed with the linearized YIp51*src* plasmid and YPH499 was transformed with the linearized YIp352*src* plasmid. The integration of YIp51*src* at the *LEU2* locus and of YIp352*src* at the *URA3* locus was confirmed by restriction enzyme digestion of genomic DNA and Southern blot analysis (data not shown). Immunoblotting analysis (see below) confirmed that each integrant strain expressed pp60$^{v\text{-}src}$ in a galactose-dependent manner. Diploids containing two integrated copies of galactose-inducible v-*src* were generated by patch-mating the MATα *leu2*::*GAL1*-v-*src*/*LEU2* haploid with the MATa *ura3*::*GAL1*-v-*src*/*URA3* haploid and selecting for Leu$^+$Ura$^+$ prototrophs.

To express human phosphotyrosine phosphatase 1B under the control of the galactose-inducible *GAL1* promoter, the 1840 bp *Bgl* I-*Sau* I fragment encoding the human PTP1B gene (kindly provided by David Hill, Oncogene Science) was inserted by blunt end ligation into the *Bam*HI site of YEp24-G (gift of B. Benton), a high-copy yeast expression vector. YEp24-G was constructed by replacing the 276 bp *Sal* I-*Bam* HI fragment of YEp24 (Botstein *et al.*, 1979) with the 673 bp *Sal* I-*Bam* HI fragment of the yeast *GAL1* promoter (Johnston and Davis, 1984).

Immunoblotting and Immune-Complex Kinase Assays

Lysates of yeast expressing v-*src* were prepared as follows. The cells were grown to late exponential phase (~10^7/ml) at 30°C in minimal medium containing 2% (wt/vol) raffinose. Expression of v-*src* was induced by addition of galactose (2%). After incubation for 3.5 h, cells from a 100-ml sample of the culture were collected by centrifugation, resuspended in 150 mM NaCl, 5 mM EDTA, 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.2, buffer and sedimented again. The washed cell pellet was resuspended in an equal volume of radio-immune precipitation assay (RIPA) buffer (150 mM NaCl, 1% wt/vol) sodium deoxycholate, 1% (wt/vol) Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 7.2) containing 0.1 mM Na_3VO_4, and the protease inhibitors antipain, leupeptin, pepstatin, aprotinin, and chymostatin (5 μg/ml each) and were then lysed by the glass bead method (Kornbluth *et al.*, 1987); lysates were clarified by centrifugation at 15 000 × *g* for 10 min.

To determine the level of protein-tyrosine phosphorylation and the level of v-*src* expression, clarified extracts were analyzed by immunoblotting as described (Liebl *et al.*, 1992), using either an antiphosphotyrosine monoclonal antibody (mAb 4G-10) (Upstate Biotechnology, Lake Placid, NY) or an anti-*src* monoclonal antibody (mAb 2-17) (Microbiological Associates, Rockville, MD) directed against the amino terminus of pp60^{src}. Lysates (100–200 μg total protein) were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene-difluoride (PVDF) membranes and incubated with the appropriate antibody. Blocking buffer (3% dry milk, 0.5% bovine serum albumin [BSA], 0.1% Tween 20, 0.5% NaCl, 0.5% Nonidet P-40, and 50 mM Tris-HCl [pH 7.5]) was used for all antibody incubation and rinsing steps; however when antiphosphotyrosine mAb was the primary antibody used, milk was omitted from the blocking buffer and the BSA concentration was increased to 3%. After incubation with primary antibody and rinsing, immunoblots were incubated either with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Pierce, Rockford, IL), which was detected using a chemiluminescence detection system (Amersham, Arlington Heights, IL) and quantified by densitometry, or with ^{125}I-protein A, which was detected and quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

An immune complex kinase assay was used to assess the activity of pp60^{src}, essentially as described (Liebl *et al.*, 1992). In brief, equal amounts of protein (3 mg) were immunoprecipitated using mAb 2-17. The immunoprecipitates were divided into two portions; one portion was used to quantify the amount of pp60^{src} in the immunoprecipitate by immunoblotting, while the other portion was used for immune-complex kinase assays using acid-denatured enolase (10 μg per 30 μl reaction) as the exogenous substrate. Incorporation of radiolabel was quantified using the Phosphorimager.

Analysis of Cdc28p Phosphorylation and Kinase Activity

To examine the level of of *Cdc28p* and its phosphorylation at tyrosine, a strain of yeast (204-4-C [gift of R. Deshaies]) was used that contains an integrated copy of *CDC28* tagged with an epitope (YPYDVPDYA) of influenza virus hemagglutinin (HA). Strain Scy33, which contains an integrated copy of the Y19F mutant of *CDC28*-HA and was derived from the same strain background, was kindly provided by B. Booher. The cells were broken in a lysis buffer 100 mM NaCl, 50 mM β-glycerophosphate [pH 7.4], 5 mM EDTA, 2 mM dithiothreitol (DTT) and 0.2% Triton X-100, plus protease inhibitors (as described above) and *Cdc28*p-HA was immunoprecipitated with a monoclonal antibody directed against the HA epitope (mAb 12 CA5 [BABCO]) (Wilson *et al.*, 1984). The immunoprecipitates were analyzed by immunoblotting with anti-HA and anti-phosphotyrosine monoclonal antibodies.

To assay the kinase activity of Cdc28p, cell lysates (2 mg total protein) were incubated with an agarose conjugate of a glutathione-S-transferase fusion protein containing full length *S. pombe* p13^{suc1} (Upstate Biotechnology; 150 μg protein per reaction). The beads were washed three times in lysis buffer and twice in reaction buffer {20 mM *N*-2-hydroxyethylpiperazine-*N*'-ethanesulfonic acid (Hepes) [pH 7.2] 10 mM $MgCl_2$, 2 mM EDTA, 2 mM dithiothreitol, and 0.02% Triton X-100}, then divided into separate portions for kinase assays and immunoblot analysis with anti-HA mAb. Kinase reaction mixtures

Table 1. Expression and toxicity of v-*src* mutants in yeast

Mutant	Mutation[a]	Relative level of p60[b]	p60 kinase activity[c]	Viable titer[d]
Vector	—	0	0	0.99
Wild-type src	None	[100]	[100]	0.00
K295M	K295M	50	0	0.96
MM4	M1MDL	128	77	0.16
XD4	Δ77-225/DLE	98	180	0.81
XD6	Δ149-174/SRG	68	175	0.84
SPX1	Q225QLE	83	110	0.83
SHX13	A228ARA	91	82	0.82
SRX5	Y416SRD	106	72	0.00

[a] Substitution or insertion mutations are given as XNY, indicating that amino acid X at position N is substituted by amino acid(s) Y; linker insertion-deletion mutations are given as ΔN_1-N_2/XYZ, indicating that the segment from residue N_1 to residue N_2 is deleted and replaced by amino acids XYZ.

[b] Based on the total level of $pp60^{v\text{-}src}$ determined by immunoblotting.

[c] Based on determinations of the specific activity of $pp60^{v\text{-}src}$ in immune complex kinase assays.

[d] Ratio of the number of viable cells recovered on medium containing galactose relative to the number of viable cells recovered on medium containing glucose.

(10 μl) contained 10% of the $p13^{suc1}$ beads in reaction buffer supplemented with 1.5 μg histone H1 (Boehringer Mannheim, Indianapolis, IN) and 50 μM [γ^{32}P]ATP (specific activity 10 Ci/mM). Reaction mixtures were incubated at 30°C for 5 min, boiled in SDS sample buffer, and analyzed by SDS-PAGE. The relative specific activity of the Cdc28 kinase was obtained by dividing cpm incorporated into histone H1 by the quantity of immunoprecipitated Cdc28 protein, as determined by immunoblotting and densitometry.

Growth Rate Measurements

Yeast were grown overnight to late exponential phase (A_{600nm} = 0.5) in medium containing 2% raffinose and then diluted 1:100 in medium containing galactose. Cell growth was then monitored periodically by measuring A_{600nm}. The number of viable cells was determined by plating various dilutions of the cultures on medium containing either glucose or galactose and nutrients appropriate to select for the maintenance of plasmids.

Immunofluorescence Staining of Nuclei and Microtubules

Cells were grown to late exponential phase in selective minimal medium containing raffinose, diluted 1:100 in medium containing galactose and grown for 8 h. Cells were fixed for 2 h in 4% formaldehyde at room temperature and then washed twice in 0.1 M potassium phosphate buffer, pH 7.5. The cell walls were removed by incubation for 45 min at 37°C with 40 μg/ml Zymolayse (ICN Biomedicals, Costa Mesa, CA) and 1 μl β-mercaptoethanol. The fixed cells were then allowed to attach to glass slide wells coated with polylysine, and then treated first with methanol at −20°C for 6 min, and subsequently with acetone at −20°C for 30 s. Thereafter the cells were washed with phosphate-buffered saline (PBS) (Harlow and Lane, 1988) containing 1 mg/ml BSA (PBS/BSA) and incubated for 1 h in 1:50 dilution of rat mAb directed against yeast tubulin (YOL1/34, Accurate Chemical and Scientific Corporation, Westbury, NY). The cells were then washed with PBS/BSA and incubated for 45 min in rhodamine-conjugated goat antibody directed against rat immunoglobulin (Cappel Research Products, Durham, NC). The preparations were mounted in glycerol containing 22.5 mg/ml 4,6-diamidino-2-phenylindole (DAPI) and examined using a Zeiss epifluorescence microscope at 1000× magnification.

Determination of DNA Content by Flow Cytometry

Cells were grown to A_{600nm} = 0.5 in selective medium containing 2% raffinose and then diluted 1:100 in 25 ml medium containing 2% galactose to induce v-*src* expression. The cultures were grown at 30°C for 10 h, washed with distilled water, and then fixed in 1 ml 70% ethanol for 1 h at room temperature. The fixed cells were washed, resuspended in 200 μl 0.2 M Tris-HCl (pH 7.5), 20 mM EDTA containing 1 mg/ml boiled pancreatic RNase A (Sigma, St. Louis, MO) and incubated for 4 h at 37°C. The treated cells were washed twice in PBS and stained overnight at 4°C in 100 μl PBS containing 50 μg/ml propidium iodide. The cells were then diluted 10-fold in PBS and sonicated for 30 s. The fluorescence of the stained cells was measured with a Becton Dickinson (Mountain View, CA) FACS flow cytometer equipped with the LYSIS II software package. At least 10 000 cells were counted per sample. Parental yeast strains lacking v-*src* and the same strains grown in 1 μM α factor, 10 mM hydroxyurea, or 80 μg/ml nocodazole were used as controls.

RESULTS

Expression of Wild-Type and Mutant v-src in Yeast

It has been shown previously that expression of v-*src* in *S. cerevisiae* is lethal (Brugge *et al.*, 1987; Kornbluth *et al.*, 1987). To examine the mechanism by which v-*src* arrests growth, wild-type v-*src* and mutants of v-*src* with alterations in the catalytic, *src* homology (SH2/SH3) or myristoylation domains were placed under the control of the galactose-inducible *GAL1* promoter and introduced into yeast on a high copy expression vector. The mutants used and the mutations they contain are listed in Table 1. Following galactose induction, the level of $pp60^{src}$ expression was monitored by immunoblotting with anti-$pp60^{src}$ monoclonal antibody and the immune-complex kinase activity of $pp60^{src}$ was monitored using denatured enolase as exogenous substrate (Table 1). All of the mutant proteins were expressed at levels comparable to the level at which wild-type protein was ex-

pressed. As expected, the mutant K295M, with an amino acid substitution within the ATP-binding site of pp60^{src}, was catalytically inactive in the immune-complex kinase assay, whereas all the other mutant proteins exhibited levels of kinase activity comparable to that of wild-type v-*src* (Table 1).

Effects of v-src Expression on Growth

To examine the effects of wild-type and mutant v-*src* on the growth of yeast, strains expressing the wild-type or mutant genes were grown in the presence of raffinose and then transferred to liquid medium containing 2% galactose. The subsequent growth of the cells was followed by monitoring the turbidity of the cultures (Figure 1). In addition, the effects of v-*src* expression on viability was determined by plating the cells on medium containing either glucose or galactose and scoring the number of colonies recovered after incubation at 30°C for 2 days (Table 1). Both in liquid medium and on plates, expression of wild-type v-*src* completely arrested growth (Figure 1, Table 1). In contrast, the mutant K295M, which encodes a catalytically inactive pp60^{src}, did not have any detectable effect on growth (Figure 1, Table 1). The mutant SRX5, in which Tyr416 (the major autophosphorylation site) is substituted by the sequence Ser Arg Asp, also completely inhibited growth (Figure 6, Table 1). In liquid medium, a mutant in which the myristoylation site is eliminated (MM4), or mutants with alterations in the *src* homology domains (SPX1, SHX13, XD4 and XD6), induced some growth delay (Figure 1), but eventually grew to the same final density as cells expressing an empty vector (data not shown). Correspondingly, these mutants induced only slight inhibition of growth on galactose plates (Table 1). Because the catalytically inactive mutant K295M has no observable effect on growth, we conclude that the inhibition of growth by pp60$^{v\text{-}src}$ is completely dependent on its catalytic activity. This result also indicates that neither perturbation of membrane function due to membrane attachment of v-*src* nor disruption of cytoskeletal structure due to association of v-*src* via its SH2/SH3 domains are sufficient to cause the growth inhibition. However the myristoylation and *src* homology domains contribute to the growth arrest, suggesting that these domains may be important in targeting the active kinase to the site(s) where the growth inhibitory protein phosphorylation occurs or in interactions between the kinase and tyrosine phosphorylated substrates.

Phosphorylation of Yeast Proteins by v-src

The dependence of the growth arrest on v-*src* kinase activity suggested that it is mediated by tyrosine phosphorylation of yeast proteins. To examine the proteins phosphorylated by wild-type and mutant forms of v-*src*, cells expressing the different forms of v-*src* were lysed and phosphotyrosyl proteins in the lysates were

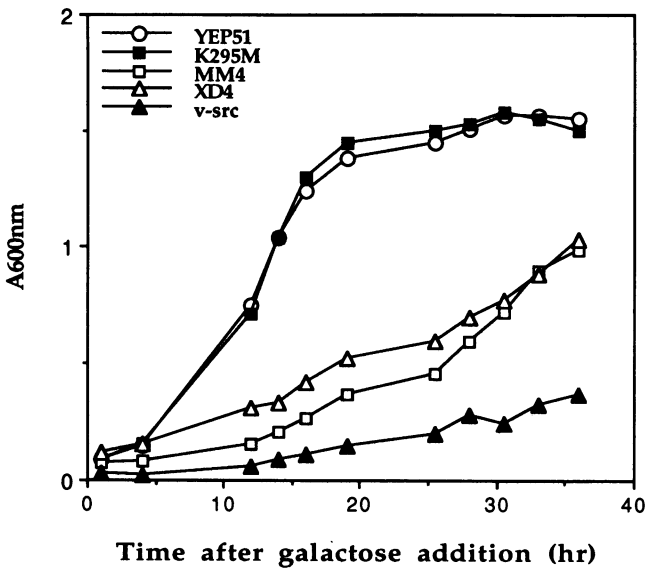

Figure 1. Growth of yeast expressing wild-type or mutant v-*src* from multi-copy plasmids. Strain YPH500 was transformed by the vector YEp51 or by YEp51 carrying the indicated inserts. Cells were grown in medium containing 2% raffinose and then diluted 1:100 in medium containing 2% galactose. Cultures were grown at 30°C and samples were removed every 2 h to measure A_{600nm}.

examined by immunoblotting with anti-phosphotyrosine antibody. A large number of phosphotyrosyl proteins were detected in cells expressing wild-type v-*src*; particularly prominent were proteins with apparent molecular masses of 40, 58, 75, and 100 kDa. Similar phosphotyrosyl proteins were detected in cells expressing the myristoylation-defective or *src* homology domain mutants, although XD4, which contains a deletion of the SH3 domain and most of SH2, induced the appearance of some additional phosphotyrosyl proteins. As expected, no phosphotyrosyl proteins were detected in cells expressing the empty vector or the catalytically inactive K295M mutant (the immunoblotting conditions used here were not sufficiently sensitive to detect endogenous yeast phosphotyrosyl proteins). Surprisingly, however, the autophosphorylation site mutant SRX5 induced only very low levels of tyrosine phosphorylation on yeast proteins in vivo (Figure 2, lane 12) despite readily detectable protein tyrosine kinase activity on enolase in vitro (Table 1). The growth arrest induced by SRX5 is dependent on tyrosine phosphorylation (see below). These observations suggest that only a minor subset of the proteins that are phosphorylated by wild-type pp60$^{v\text{-}src}$ are involved in the growth arrest.

The *CDC28* gene product, Cdc28p (or p34^{CDC28}), is phosphorylated at Tyr19 in vivo, although the effects of this tyrosine phosphorylation are unclear (Amon *et al.*, 1992; Sorger and Murray, 1992). In addition, pp60^{src} can phosphorylate p34^{CDC28} in vitro (Draetta *et al.*, 1988; Litwin *et al.*, 1991) (L. Wilson, unpublished observa-

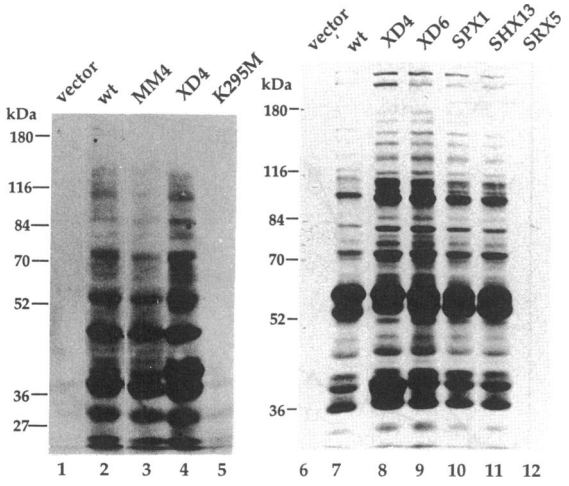

Figure 2. Phosphotyrosine-containing proteins in yeast expressing wild-type or mutant v-*src* genes. v-*src* expression was induced by addition of galactose. After 3.5 h at 30°C in medium containing galactose, the cells were lysed and 50 μg protein from each lysate was resolved by SDS-PAGE. Phosphotyrosine-containing proteins were detected by immunoblotting with anti-phosphotyrosine antibody. The 2 panels represent 2 separate experiments, both performed as described in MATERIALS AND METHODS; molecular mass standards are indicated to the left of each panel. Strain YPH500 carried the vector YEp51 with the following inserts: no insert (lanes 1, 6); wild-type v-*src* (lanes 2, 7); MM4 (lane 3); XD4 (lanes 4, 8); K295M (lane 5); XD6 (lane 9); SPX1 (lane 10); SHX13 (lane 11); and SRX5 (lane 12).

tions). To determine if phosphorylation of Cdc28p could account for v-*src*-induced growth inhibition, a plasmid containing a galactose-inducible copy of v-*src* was introduced into a strain expressing Cdc28p tagged with an influenza virus haemagglutinin (HA) epitope (Cdc28p-HA). The v-*src* expression plasmid was also introduced into a strain expressing a mutant of Cdc28p-HA in which Tyr 19 had been substituted by phenylalanine (Y19F-Cdc28p-HA). Following galactose induction of v-*src* expression, Cdc28p-HA was immunoprecipitated with a monoclonal antibody directed against the HA epitope, and the immunoprecipitates analyzed by immunoblotting with either anti-HA epitope or anti-phosphotyrosine antibodies (Figure 3). Cdc28p-HA was phosphorylated to a much greater extent in cells expressing v-*src* than in cells expressing the corresponding vector alone. The Y19F mutant of Cdc28p-HA contained higher levels of phosphotyrosine in cells expressing v-*src* than in cells expressing vector alone. In most experiments, however the level of phosphorylation of Y19F-Cdc28p-HA was lower than that of wild-type Cdc28p-HA, suggesting that Cdc28p is phosphorylated both at Tyr19 and at other sites; we cannot exclude the possibility that these additional sites become accessible due to the presence of the HA epitope tag or reside within the HA tag itself. Cells expressing Y19F-Cdc28p-HA (as the sole source of Cdc28p in the cell) were not resistant to growth arrest by v-*src*, indicating that phosphorylation of Tyr19 of Cdc28p cannot by itself be responsible for the lethal phenotype.

The *KSS1* and *FUS3* gene products are also known to be activated by phosphorylation at tyrosine in vivo, leading to G1 cell-cycle arrest. However a *kss1 fus3* deletion strain (gift of D. Ma) was also sensitive to growth arrest by v-*src* (L. Wilson, unpublished observations), indicating that growth inhibition by v-*src* is not mediated through stimulation of the pheromone response pathway via tyrosine phosphorylation of *FUS3* or *KSS1*. Consistent with this conclusion, v-*src* expression arrests the growth of MATa/MATα diploids (Figure 6).

Effect of v-src Expression on Cdc28 Kinase Activity

The observation that Cdc28p is phosphorylated at tyrosine in cells expressing v-*src* suggested that v-*src* might affect Cdc28p kinase activity. To determine whether expression of v-*src* affects Cdc28p kinase activity and if these effects might be correlated with v-*src* toxicity, we examined the level of Cdc28p-dependent histone H1 kinase activity in cells expressing Cdc28p-HA or Y19F-Cdc28p-HA and either wild-type or mutant v-*src*. Following galactose induction of v-*src* expression, Cdc28p-HA was isolated by adsorption to $p13^{suc1}$ beads and incubated with [γ-^{32}P]ATP and histone H1. Wild-type v-*src* induced a 2.5-fold increase in Cdc28p kinase activity in cells expressing Cdc28p (Figure 4). A similar increase was observed in cells expressing wild-type v-*src* and the Y19F mutant of Cdc28p (M. Florio, L. Wilson, and J. Trager, unpublished observations). This indicates that phosphorylation of Cdc28p at Tyr19, which is generally associated with a decrease in Cdc28 kinase activity, is unrelated to the activation of Cdc28p kinase activity, and probably occurs at low stoichiometry. The v-*src* mutant SRX5, which is as effective as

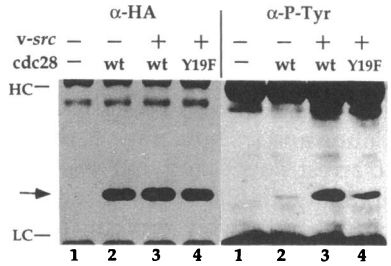

Figure 3. v-*src*-induced phosphorylation of Cdc28p. Following galactose induction of v-*src* expression, lysates were prepared and HA-epitope-tagged Cdc28p was immunoprecipitated with anti-HA mAb. The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-HA mAb (left) or anti-phosphotyrosine mAb (right). Yeast strains expressed: no exogenous gene (strain YPH500) (lane 1); CDC28-HA (strain 204-4-C) and YEp51 (lane 2); CDC28-HA and YEp51-*src* (lane 3); Y19F-CDC28-HA (strain Scy33) and YEp51-*src* (lane 4). Arrow indicates Cdc28p-HA. HC and LC indicate the positions of the anti-HA immunoglobulin heavy and light chains, respectively.

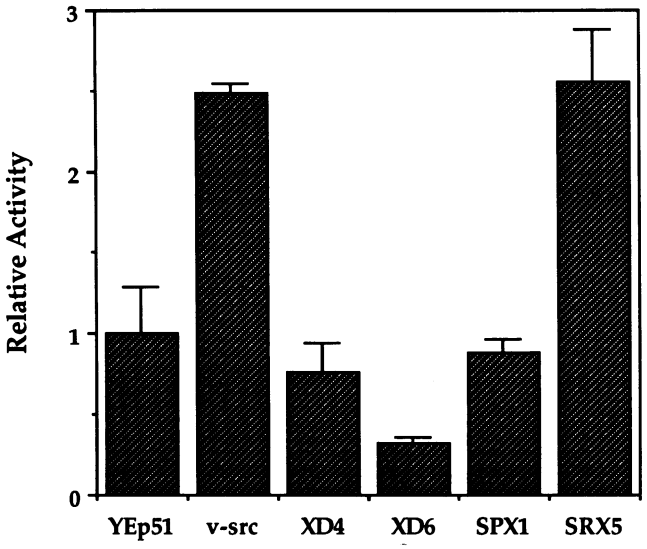

Figure 4. Activation of Cdc28p histone H1 kinase activity by v-*src*. Cdc28p was precipitated from strains expressing vector YEp51 or vector YEp51 carrying the indicated inserts. Precipitates were incubated with histone H1 and [γ-^{32}P]ATP and kinase specific activities were determined as described under MATERIALS AND METHODS. Specific activities were normalized to the value obtained for cells not expressing v-*src*; error bars represent standard deviations.

wild-type v-*src* in arresting cell proliferation, also caused an increase in H1 kinase activity whereas the SH2/SH3 mutants XD4, XD6, and SPX1 did not (Figure 4). This suggests that the increase in Cdc28p kinase activity is correlated with the toxicity of v-*src*.

Reversal of v-src Toxicity by a Mammalian Protein Tyrosine Phosphatase

If v-*src* toxicity is mediated by tyrosine phosphorylation of certain yeast proteins, it might be reversed by expression of an exogenous phosphotyrosine-specific phosphoprotein phosphatase. To examine this possibility, strains containing a single integrated copy of a galactose-inducible wild-type v-*src* gene were transformed with a high copy expression plasmid containing the human placental protein tyrosine phosphatase 1B (PTP1B) gene under the control of the *GAL1* promoter. One hundred thirteen out of 140 independent transformants were able to grow on medium containing galactose, although growth was somewhat reduced compared with strains expressing only PTP1B or the vector alone (Figure 5, panels A and C). When the PTP1P plasmid from the v-*src* resistant transformants was removed by counterselection with 5-fluoro-orotic acid, (Boeke *et al.*, 1984) the cells reverted to their previous galactose-sensitive state (L. Wilson, unpublished observations), indicating that rescue from galactose-induced v-*src* toxicity requires the continued maintenance of the PTP1B plasmid. To determine the effects of PTP1B expression on the level of protein-tyrosine phosphorylation, lysates of cells expressing v-*src* in the presence or absence of PTP1B expression were analyzed by immunoblotting with anti-phosphotyrosine antibody (Figure 5B). Expression of PTP1B greatly reduced, but did not completely eliminate, the tyrosine phosphorylation of yeast proteins; this finding is consistent with the observation that PTP1B expression reduced, but did not completely overcome, the toxicity of v-*src*. These findings further support the conclusion that it is the tyrosine phosphorylation of certain yeast proteins that is responsible for the v-*src* toxicity.

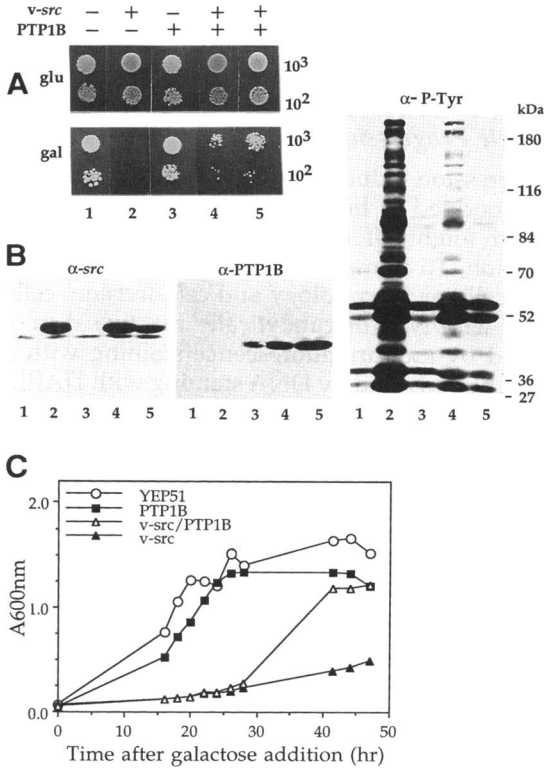

Figure 5. Rescue from v-*src* lethality by human phosphotyrosyl-phosphatase hPTP1B. Panel A, viability of yeast expressing v-*src* in the presence or absence of PTP1B. Cells (10^2 or 10^3) were applied to selective plates containing glucose or galactose and incubated for 2 d (glucose) or 4 d (galactose). Strains used were: YPH500 expressing vector YEp24G (column 1); galactose-inducible v-*src* *LEU2* integrant strain (YPH500 *leu2::GAL1*-v-*src*/LEU2) transformed with vector YEp24G (column 2); YPH500 expressing YEp24G-hPTP1B (column 3); 2 independent transformants of the YPH500 v-*src* integrant with YEp24G-hPTP1B (columns 4, 5). Panel B, effects of PTP1B expression on tyrosine phosphorylation induced by v-*src*. Each lane represents 200 μg cell protein separated by SDS-PAGE and immunoblotted with mAb directed against either *src*, PTP1B or phosphotyrosine, as indicated. Lane numbers correspond to column numbers in (A). Panel C, growth of yeast expressing v-*src* in the presence or absence of PTP1B. Cells were grown as described in the legend to Figure 1. Strains used were YPH500 transformed with: vector YEp51 (○); YEp24G-hPTP1B (■); YEp51-v-*src* (▲); and both YEp51-v-*src* and YEp24G-hPTP1B (△).

As noted above, the level of tyrosine phosphorylation in cells expressing the autophosphorylation site mutant SRX5 was very low, even though SRX5 completely inhibited cell proliferation. To determine if the lethality of SRX5 was dependent on tyrosine phosphorylation, cells were transformed with both *GAL1*-SRX5*src* and *GAL1*-PTP1B. The growth of these cells in medium containing galactose was compared with that of cells expressing *GAL1*-SRX5*src* alone (Figure 6). PTP1B restored the ability of cells expressing *GAL1*-SRX5*src* to proliferate in medium containing galactose, both on plates (Figure 6A) and in liquid medium (Figure 6B), although, as with wild-type v-*src*, the reversal of lethality was not complete. We conclude that the lethality of SRX5*src*, like that of wild-type *src*, involves tyrosine phosphorylation of yeast proteins.

Effects of v-src Expression on Cell Morphology and Cell-Cycle Progression

v-*src* expression induced the appearance of abnormally large unbudded or budded cells (Figure 7) suggesting that v-*src* might affect cell division and/or cell cycle progression. To characterize the effects of v-*src* expression on cellular morphology and cell division, cells expressing wild-type or catalytically inactive v-*src* were examined by immunofluorescence staining with anti-tubulin antibody and by DNA staining with DAPI. The phenotype of cells expressing wild-type v-*src* was quite heterogeneous. Cells expressing wild-type v-*src* exhibited a number of alterations in microtubule distribution, including the presence of multiple spindles that were separated from their nuclear attachment sites and disoriented with respect to the daughter cells (Figure 7, panels b,c). DAPI staining revealed large anucleate unbudded cells, budded cells with the nucleus retained at the bud neck, and binucleate or multinucleate cells with prominent punctate (presumably mitochondrial) staining (Figure 7, panels g,h). No morphological abnormalities were observed in cells expressing the catalytically inactive K295M mutant (Figure 7, panels d,i), indicating that these changes are dependent on the catalytic activity of the *src* kinase. The heterogeneous phenotype of cells expressing v-*src* was probably not due to heterogeneity in the level of *src* expression, since this heterogenous phenotype was observed in a diploid strain carrying two integrated copies of galactose-inducible v-*src* (Figure 7, panels b, c, g, h). Cells expressing the autophosphorylation site mutant SRX5 displayed morphological abnormalities similar to those observed in cells expressing wild-type *src* (Figure 7, panels e, j), indicating that although the level of tyrosine phosphorylation induced by SRX5*src* is much lower than that induced by wild-type *src*, the mutant kinase induces the same phenotype as the wild type.

The observations described above suggested that the toxicity of v-*src* in yeast might be associated with abnormalities in microtubule distribution and function. To characterize further the effects of v-*src* on microtubule function, cells expressing wild-type or mutant v-*src* were grown under repressing or inducing conditions in the presence of increasing concentrations of the microtubule-destabilizing drug Benomyl. Induction of v-*src* increased the sensitivity of the cells to inhibition of growth by Benomyl. For example cells expressing XD4, which induces partial inhibition of growth, were sensitive to 5–10 μg/ml Benomyl, whereas cells expressing vector alone or the K295M mutant were sensitive to growth inhibition by Benomyl only at concentrations above 25–30 μg/ml (Figure 8). These observations are consistent with the idea that growth inhibition by v-*src* is associated with, and may result from, abnormalities in microtubule function.

To examine the effects of v-*src* expression on cell-cycle progression, exponentially growing cells carrying

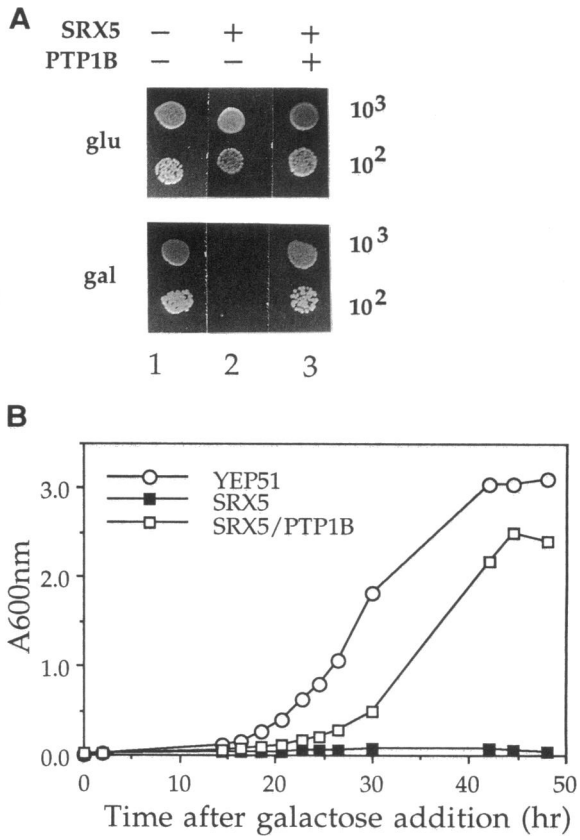

Figure 6. Toxicity of SRX5*src* and its reversal by human phosphotyrosyl-phosphatase hPTP1B. (A) Viability of yeast expressing SRX5*src* in the presence or absence of PTP1B. Cells were applied to selective plates containing glucose or galactose as described in the legend to Figure 5A. Strains used were YPH500 transformed with: vector YEp51 (column 1); YEp51-SRX5 (column 2); and both YEp51-SRX5 and YEp24G-hPTP1B (column 3). (B) Growth of yeast expressing SRX5*src* in the presence or absence of PTP1B. Cells were grown as described in the legend to Figure 1. The strains used were the same as those shown in A, as indicated.

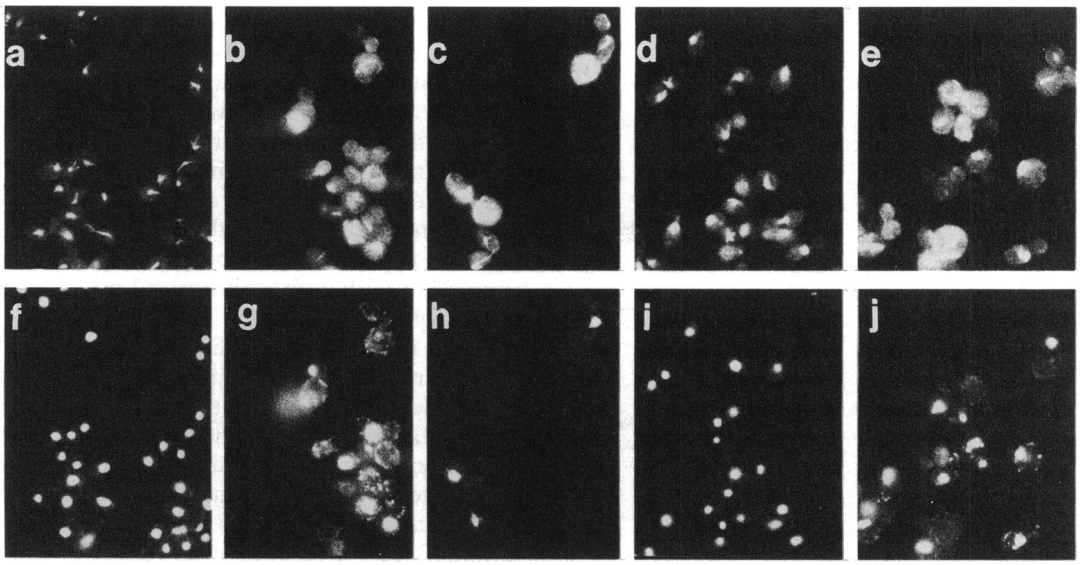

Figure 7. Effects of v-*src* expression on microtubule distribution and nuclear morphology. Diploid yeast (strain YPH501) were grown in raffinose minimal medium, diluted 1:100 in medium containing galactose and grown for 8 h. Microtubules were stained using rat anti-tubulin mAb and rhodamine-conjugated anti-rat Ig (panels a–e) and nuclei were stained with DAPI (panels f–j). Panels represent fluorescence micrographs of YPH501 expressing vector YEp51 (panels a, f); YPH501 containing 2 integrated copies (leu2::*GAL1*-v-*src*/*LEU2 ura3*::*GAL1*-v-*src*/*URA3*) of galactose-inducible v-*src* (panels b, c, g, h); YPH501 expressing YEp51-K295M*src* (panels d, i); and YPH501 expressing YEp51-SRX5*src* (panels e, j).

GAL1-regulated v-*src* on a high copy plasmid or integrated into the genome were induced with galactose and the DNA content of the cells was determined by propidium iodide staining and flow cytometry. Cells expressing vector alone or catalytically inactive *src* contained roughly equal numbers of G1 and G2 phase cells. Induction of plasmid-borne or integrated v-*src* led to an increase in the fraction of cells with a G1 (1C) DNA content and to the appearance of cells with a DNA content of less than 1C or greater than 2C (Figure 9). These observations suggest that *src* kinase activity causes abnormalities either in the replication of DNA or its segregation at mitosis, or both. Induction of SRX5*src* also led to the appearance of cells with a DNA content greater than 2C (Figure 9), confirming that expression of this mutant induces the same phenotype as wild-type v-*src*.

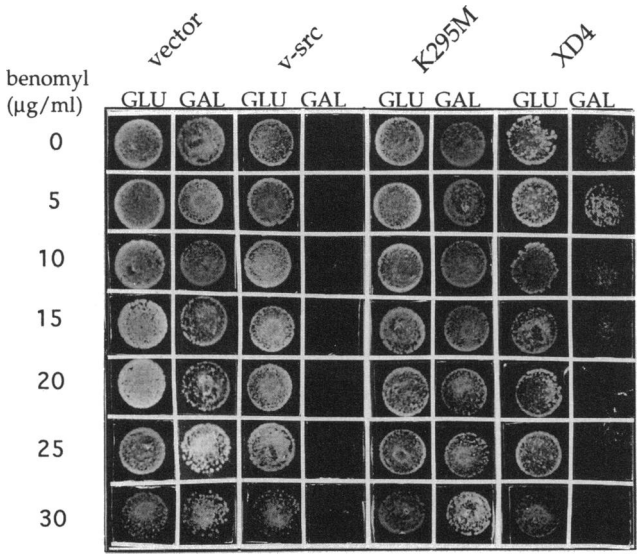

Figure 8. Effects of *src* expression on sensitivity to Benomyl. Cell suspensions were diluted 1:30 in sterile water and spotted onto selective plates containing glucose or galactose and the indicated amount of Benomyl. Strain YPH500 expressed empty vector YEp51, YEp51-v-*src*, Yep51-K295M*src*, or YEp51-XD4*src*, as indicated.

DISCUSSION

The Tyrosine Kinase Activity of pp60$^{v\text{-}src}$ Is Required for Growth Arrest

The observations reported here indicate that the growth arrest and morphological alterations induced by v-*src* expression in budding yeast are dependent on the catalytic activity of the v-*src* kinase. The expression of an active v-*src* kinase leads to the phosphorylation of a variety of proteins at tyrosine. The growth arrest, the morphological alterations and the tyrosine phosphorylation of yeast proteins were all reversed by co-expression with a mammalian phosphotyrosine phosphatase. These findings indicated that the growth arrest is due to the phosphorylation of certain yeast proteins at tyrosine. This conclusion is consistent with the observation that the toxicity of v-*src* is relieved by deletion of the

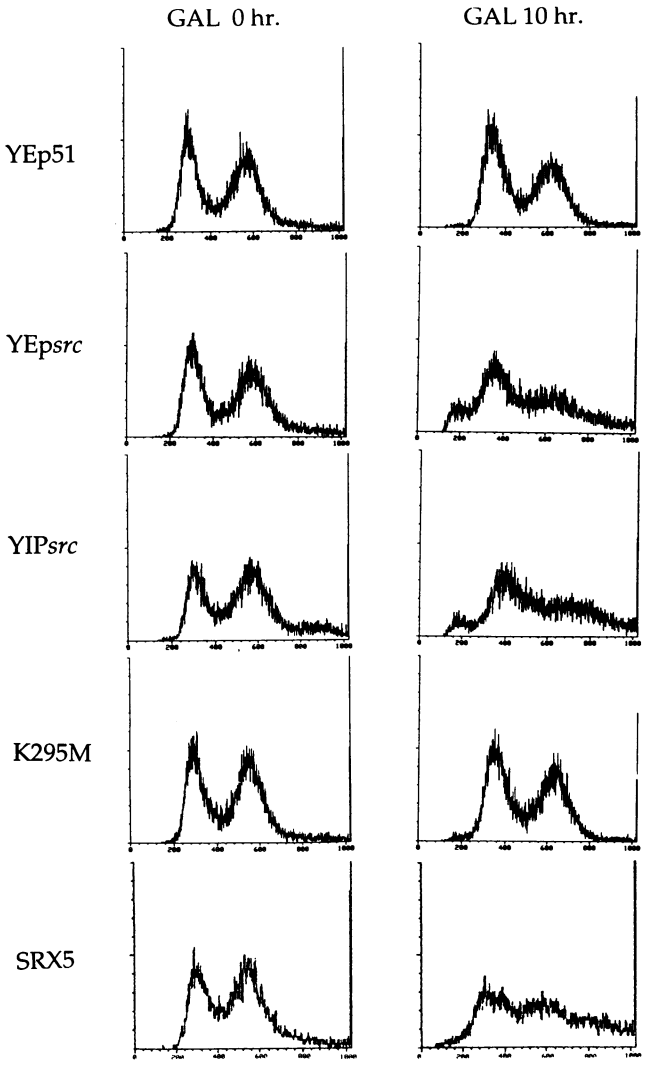

Figure 9. DNA content of cells expressing wild-type or catalytically inactive v-*src*. Cells were grown in minimal medium containing raffinose, and *src* expression was induced by addition of galactose. Cells were fixed and stained with propidium iodide either at the time of addition of galactose (left panels) or after 10-h growth at 30°C (right panels). DNA content was determined by flow cytometry. Panels show strain YPH500 expressing: vector YEp51; YEp51-v-*src*; YIp*src*, an integrated copy of galactose-inducible v-*src* (*leu2::GAL1*-v-*src*/*LEU2*); YEp51-K295M*src*; and YEp51-SRX5*src*.

yeast heat shock gene Hsc82, and that this mutation reduces both the kinase activity of pp60v-*src* and its level of expression (Xu and Lindquist, 1993). It is also consistent with the finding that the inhibition of yeast cell proliferation resulting from expression of c-*src* can be reversed by co-expression of a regulatory kinase that inhibits c-*src* kinase activity (Murphy *et al.*, 1993; Superti-Furga *et al.*, 1993).

In a number of respects these findings parallel those made on expression of v-*src* in animal cells. Expression of v-*src* in susceptible cells generally results in transformation, but high levels of *src* expression are known to be toxic (Tarpley and Temin, 1984). Transformation of animal cells is dependent on the catalytic activity of $pp60^{v\text{-}src}$ and, like the growth arrest observed in yeast, is promoted by the myristoylation and SH2/SH3 domains. In avian or mammalian cells, $pp60^{v\text{-}src}$ phosphorylates a large number of distinct substrate proteins. Both transformation and the tyrosine phosphorylation of cellular proteins can be partially inhibited by co-expression with a phosphotyrosyl protein phosphatase (Woodford-Thomas *et al.*, 1992).

As yet no tyrosine-specific kinases have been detected in budding yeast, although protein-tyrosine phosphorylation dependent on dual specificity kinases does occur. The parallelism between the growth inhibition observed in yeast and the transformation observed in mammalian cells raises a number of questions, such as the role of the noncatalytic region of $pp60^{v\text{-}src}$ (the myristoylation and SH2/SH3 domains) in the growth arrest, and the relationship between tyrosine phosphorylation and the changes in morphology and DNA content. A central question is the identity of the substrate proteins whose phosphorylation is responsible for the growth arrest, and whether the tyrosine phosphorylation of these substrates plays a role in normal growth regulation.

The Role of the Noncatalytic Region of $pp60^{v\text{-}src}$ in Growth Inhibition

The MM4 mutation leads to a reduction in the growth inhibitory properties of v-*src*. In animal cells this mutation, and other alterations within the seven N-terminal residues of $pp60^{v\text{-}src}$, block the myristoylation of $pp60^{v\text{-}src}$ and its attachment to cellular membranes, and render the protein transformation-defective. These observations have been interpreted as indicating that transformation of animal cells by $pp60^{v\text{-}src}$ is dependent on the phosphorylation of membrane-associated substrates, or alternatively that membrane association of $pp60^{src}$ is necessary for the formation of some type of signaling complex. In budding yeast protein myristoylation is catalyzed by the NMT1 gene product (Duronio *et al.*, 1989). It seems likely therefore that the growth arrest induced by v-*src* in yeast is at least partially dependent on myristoylation of $pp60^{v\text{-}src}$ and its targeting to cellular membranes. Thus one or more of the targets of $pp60^{src}$ may be membrane associated, or, as discussed below, may become membrane-associated as the result of interactions between phosphotyrosyl residues and the SH2 domain of $pp60^{v\text{-}src}$.

The toxicity of v-*src* was reduced by linker mutations or deletions in SH2, including mutations that fall entirely within this region. Although some of the mutant *src* proteins used in this study exhibited reduced kinase activity, this reduction in kinase activity was not solely responsible for the decreased toxicity, since toxicity was observed in other strains that expressed reduced levels

of kinase activity (for example in cells expressing the mutant SRX5 on a multi-copy plasmid, or cells expressing a single integrated copy of wild-type v-*src*). In metazoan cells, SH2 domains bind to sequences that contain phosphotyrosine residues and are involved in protein-protein interactions that may be intermolecular or intramolecular. These interactions are affected by the sequence context of the phosphotyrosine residue, and the *src* SH2 domain interacts most avidly with the sequence Phosphotyrosine-Glu-Glu-Ile (Songyang *et al.*, 1993). However SH2 domains may also interact with sequences that do not contain a phosphotyrosine residue (Muller *et al.*, 1992). In animal cells mutations within the v-*src* SH2 domain may affect the intracellular targeting or substrate specificity of $pp60^{v\text{-}src}$. Similarly the effect of SH2 mutations on the ability of v-*src* to arrest the growth of yeast might result from alterations in the intracellular targeting or substrate specificity of $pp60^{src}$ (Fukui *et al.*, 1991; Liebl *et al.*, 1992). The growth arrest might also result, at least in part, from the formation of a complex between $pp60^{v\text{-}src}$ and one or more of its phosphorylated substrates, generating a steric block to the function of the complexed substrate. Indeed, in animal cells overexpressing $pp60^{c\text{-}src}$ or $pp60^{v\text{-}src}$, complexes between $pp60^{src}$ and its phosphorylated substrates can be detected (Jove *et al.*, 1987). As yet, SH2 domains have not been definitively identified in any yeast proteins but sequence analysis suggests that the nuclear protein Spt6 may contain an SH2 domain (Maclennan and Shaw, 1993). If an SH2-dependent interaction of $pp60^{v\text{-}src}$ with a phosphorylated substrate is involved in the growth arrest, the sequences interacting with the SH2 domain of $pp60^{src}$ might be sequences whose normal function does not involve an interaction with an SH2 domain, and in this case the interaction with $pp60^{src}$ would be fortuitous. However if SH2 domains do occur in some yeast proteins, the involvement of the *src* SH2 domain in the growth arrest might reflect an interaction that occurs in normal yeast cells.

Effects of v-src Expression on Cytoskeletal and Nuclear Morphology

In animal cells $pp60^{v\text{-}src}$ expression leads to disruption of the cytoskeleton and the transmembrane linkages between the cytoskeleton and extracellular matrix. These alterations affect both the microfilament (actin) and microtubule (tubulin) systems (Wang and Goldberg, 1979) and are associated with phosphorylation of a variety of cytoskeletal components, including focal adhesion kinase, vinculin, cortactin, and talin (Kellie *et al.*, 1991). In addition $pp60^{c\text{-}src}$ is known to be localized in endosomes that are clustered around microtubule organizing centers (David-Pfeuty and Nouvian-Dooghe, 1990; Kaplan *et al.*, 1992). It is possible that the cytoskeletal alterations observed in yeast expressing v-*src* could be due to the phosphorylation of cytoskeletal components. This is consistent with the finding that no cytoskeletal changes were observed in cells expressing catalytically inactive v-*src*. If phosphorylation of cytoskeletal components is responsible for the cytoskeletal alterations, the reduced toxicity of mutants with alterations in the noncatalytic domain of $pp60^{v\text{-}src}$ might result from a reduction in the level of $pp60^{v\text{-}src}$ that is targeted to the cytoskeleton. The alterations in DNA content, including the accumulation of anucleate cells and cells with a greater than 2C DNA content, could be due to abnormalities in DNA segregation resulting from defects in spindle function at mitosis. These alterations in DNA content could also result from defects in DNA replication or a defect in the checkpoint control mechanisms (Murray, 1992) that ensure an orderly alternation of S and M phases. Such defects, and the alterations in microtubule morphology, might also result, at least in part, from the elevation in $p34^{CDC28}$ kinase activity (see below).

Targets of v-src in Yeast

It is clear that the mechanism by which $pp60^{v\text{-}src}$ arrests growth involves phosphorylation of certain yeast proteins. To understand the mechanism of the growth arrest, therefore, it will be necessary to identify the proteins phosphorylated by $pp60^{v\text{-}src}$ that are the targets responsible for growth inhibition. In animal cells $pp60^{v\text{-}src}$ expression results in phosphorylation of a large number of substrates, including some that are also phosphorylated in cells stimulated by growth factors. It is generally believed that only a subset of these cellular substrates are regulated by tyrosine phosphorylation and involved in transformation, while others are phosphorylated adventitiously. Similarly in yeast expressing v-*src* it appears likely that only some of the proteins phosphorylated by $pp60^{src}$ are involved in the inhibition of growth. For example the *CDC28* protein is phosphorylated at tyrosine in cells expressing v-*src*, probably at both Tyr19 and other sites; this phosphorylation may result from direct phosphorylation by $pp60^{v\text{-}src}$, or from phosphorylation by a kinase that is activated by v-*src* expression, or both. However substitution of the Tyr19 site, the major site for tyrosine phosphorylation in normal cells, does not affect the cellular susceptibility to v-*src* toxicity. Since phosphorylation of Tyr19 is believed to result in a decrease in Cdc28 kinase activity, but expression of v-*src* increases Cdc28 kinase activity, it seems likely that the stoichiometry of Tyr19 phosphorylation by v-*src* is low and that Tyr19 phosphorylation is unrelated to the lethal phenotype. The activation of Cdc28 kinase activity might result either from Tyr phosphorylation at sites other than Tyr19, from increased Thr phosphorylation by a cdc2/*CDC28* activating enzyme (Solomon *et al.*, 1993) or from an alteration in cyclin association.

While this manuscript was in preparation, Boschelli and his colleagues (Boschelli, 1993; Boschelli *et al.*, 1993)

reported similar studies on the effects of v-*src* expression in budding yeast. In general our observations and conclusions are in substantial agreement with those reported by Boschelli *et al.* (Boschelli, 1993; Boschelli *et al.*, 1993), in particular with respect to the effects of v-*src* expression on microtubule morphology and the requirement for v-*src* SH2 function. However Boschelli *et al.* (1993) attribute the lethality of v-*src* expression primarily to mitotic catastrophe resulting from the elevation in Cdc28 kinase activity. Our findings indicate that a significant fraction of cells arrest with a G_1 (1C) content of DNA following v-*src* induction. Moreover expression of Y527F-c-*src* elevates Cdc28 kinase activity even though Y527F-c-*src* is only partially toxic (J. B. Trager, unpublished observations). These findings suggest that factors other than the elevation in Cdc28 kinase activity probably also play a role in the arrest of cell proliferation.

SRX5, which arrests growth as effectively as wild-type *src* and induces the same morphological changes, nevertheless induces only very low levels of tyrosine phosphorylation. In contrast the toxicity of wild-type v-*src* is relieved by coexpression of a phosphotyrosine phosphatase at levels which reduce, but do not eliminate, the tyrosine phosphorylation of yeast proteins. These observations suggest that SRX5 may phosphorylate only a limited subset of the proteins phosphorylated by wild-type *src*, including those that are involved in the growth arrest, and is consistent with the idea that many of the proteins phosphorylated by wild-type $pp60^{v\text{-}src}$ are not involved in the inhibition of cellular proliferation. We have recently observed that the proteins phosphorylated at tyrosine in cells expressing SRX5-*src* can be recovered by affinity chromatography on a matrix coupled to a bacterially expressed *src* SH2 domain and that these phosphotyrosyl proteins are present in reduced abundance in cells expressing *src* SH2 mutants (J. B. Trager, unpublished observations). These proteins may represent phosphorylation substrates that are involved in *src* toxicity. The identification of these targets should make it possible to determine whether the proteins phosphorylated in cells expressing v-*src* are normally regulated by phosphorylation at tyrosine and whether they are related to any substrates of $pp60^{v\text{-}src}$ present in animal cells.

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REFERENCES

Amon, A., Surana, U., Muroff, I., and Nasmyth, K. (1992). Regulation of $p34^{CDC28}$ tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. Nature *355*, 368–371.

Boeke, J.D., LaCroute, F., and Fink, G.R. (1984). A positive selection for mutants lacking orotine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. & Gen. Genet. *197*, 345–346.

Bolen, J.B., Rowley, R.B., Spana, C., and Tsygankov, A.Y. (1992). The Src family of tyrosine protein kinases in hemopoietic signal transduction. FASEB J. *6*, 3403–3409.

Boschelli, F. (1993). Expression of $p60^{v\text{-}src}$ in *Saccharomyces-cerevisiae* results in elevation of p34cdc28 kinase activity and release of the dependence of DNA replication on mitosis. Mol. Cell. Biol. *13*, 5112–5121.

Boschelli, F., Uptain, S.M., and Lightbody, J.J. (1993). The lethality of $p60^{v\text{-}src}$ in *Saccharomyces-cerevisiae* and the activation of p34 (cdc28) kinase are dependent on the integrity of the SH2 domain. J. Cell Sci. *105*, 519–528.

Botstein, D., Falco, S.C., Stewart, S.E., Brennan, M., Scherer, S., Stinchcomb, D.T., Struhl, K., and Davis, R.W. (1979). Sterile host yeast (SHY) a eukaryotic system of biological containment for recombinant DNA experiments. Gene *8*, 17–24.

Broach, J.R., Li, Y.-Y., Wu, L.-C.C., and Jayaram, M. (1983). Vectors for high-level, inducible expression of cloned genes in yeast. In: Experimental Manipulation of Gene Expression. Academic Press: New York 83–117.

Brott, B.K., Decker, S., O'Brien, M.C., and Jove, R. (1991). Molecular features of the viral and cellular Src kinases involved in interactions with the GTPase-activating protein. Mol. Cell. Biol. *10*, 5059–5067.

Brugge, J.S., Jarosik, G., Andersen, J., Queral-Lustig, A., Fedor-Chaiken, M., and Broach, J.R. (1987). Expression of Rous sarcoma virus transforming protein, $pp60^{v\text{-}src}$ in *Saccharomyces cerevisiae* cells. Mol. Cell. Biol. *7*, 2180–2187.

Chenevert, J., Corrado, K., Bender, A., Pringle, J., and Herskowitz, I. (1992). A yeast gene (BEM1) necessary for cell polarization whose product contains two SH3 domains. Nature *356*, 77–79.

Cooper, J.A., and Howell, B. (1993). The when and how of Src regulation. Cell *73*, 1051–1054.

David-Pfeuty, T., and Nouvian-Dooghe, Y. (1990). Immunolocalization of the cellular *src* protein in interphase and mitotic NIH c-*src* overexpresser cells. J. Cell Biol. *111*, 3097–3116.

DeClue, J.E., and Martin, G.S. (1989). Linker insertion-deletion mutagenesis of the v-*src* gene: isolation of host- and temperature-dependent mutants. J. Virol. *63*, 542–554.

Draetta, G., Piwnica-Worms, H., Morrison, D., Druker, B., Roberts, T., and Beach, D. (1988). Human cdc2 protein kinase is a major cell-cycle regulated tyrosine kinase substrate. Nature *336*, 738–744.

Drubin, D.G., Mulholland, J., Zhu, Z., and Botstein, D. (1990). Homology of a yeast actin-binding protein to signal transduction proteins and myosin-I. Nature *343*, 288–290.

Duronio, R.J., Towler, D.A., Heuckeroth, R.O., and Gordon, J.I. (1989). Disruption of the yeast N-myristoyl transferase gene causes recessive lethality. Science *243*, 796–800.

Errede, B., Gartner, A., Zhou, Z., Nasmyth, K., and Ammerer, G. (1993). MAP kinase-related FUS3 from *S. cerevisiae* is activated by STE7 in vitro. Nature *362*, 261–264.

Featherstone, C., and Russell, P. (1991). Fission yeast $p107^{wee1}$ mitotic inhibitor is a tyrosine/serine kinase. Nature *349*, 808–811.

Fukui, Y., O'Brien, M.C., and Hanafusa, H. (1991). Deletions in the SH2 domain of $pp60^{v\text{-}src}$ prevent association with the detergent-insoluble cellular matrix. Mol. Cell. Biol. *11*, 1207–1213.

Gartner, A., Nasmyth, K., and Ammerer, G. (1992). Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. Genes & Dev. *6*, 1280–1292.

Guan, K., Deschenes, R.J., and Dixon, J.E. (1992a). Isolation and characterization of a second protein tyrosine phosphatase gene, *PTP2*, from *Saccharomyces cerevisiae*. J. Biol. Chem. *267*, 10024–10030.

Guan, K., Deschenes, R.J., Qiu, H., and Dixon, J.E. (1991). Cloning and expression of a yeast protein tyrosine phosphatase. J. Biol. Chem. *266*, 12964–12970.

Guan, K., Hakes, D.J., Wang, Y., Park, H.-D., Cooper, T.G., and Dixon, J.E. (1992b). A yeast protein phosphatase related to the vaccinia virus VH1 phosphatase is induced by nitrogen starvation. Proc. Natl. Acad. Sci. USA *89*, 12175–12179.

Hirai, H., and Varmus, H.E. (1990). Site-directed mutagenesis of the SH2- and SH3-coding domains of c-*src* produces varied phenotypes, including oncogenic activation of $p60^{c\text{-}src}$. Mol. Cell. Biol. *10*, 1307–1318.

James, P., Hall, B.D., Whelen, S., and Craig, E.A. (1992). Multiple protein tyrosine phosphatase-encoding genes in the yeast *Saccharomyces cerevisiae*. Gene *122*, 101–110.

Johnston, M., and Davis, R.W. (1984). Sequences that regulate the divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *4*, 1440–1448.

Jove, R., Kornbluth, S., and Hanafusa, H. (1987). Enzymatically inactive $pp60^{c\text{-}src}$ mutants with altered ATP-binding site is fully phosphorylated in its carboxy-terminal regulatory region. Cell *50*, 937–943.

Kaplan, K.B., Swedlow, J.R., Varmus, H.E., and Morgan, D.O. (1992). Association of $p60^{c\text{-}src}$ with endosomal membranes in mammalian fibroblasts. J. Cell Biol. *118*, 321–333.

Kellie, S., Horvath, A.R., and Elmore, M.A. (1991). Cytoskeletal targets for oncogenic tyrosine kinases. J. Cell Sci. *99*, 207–211.

Kornbluth, S., Jove, R., and Hanafusa, H. (1987). Characterization of avian and viral $p60^{src}$ proteins expressed in yeast. Proc. Natl. Acad. Sci. USA *84*, 4455–4459.

Kumagai, A., and Dunphy, W.G. (1991). The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. Cell *64*, 903–914.

Liebl, E.C., England, L.J., DeClue, J.E., and Martin, G.S. (1992). Host range mutants of v-*src:* alterations in kinase activity and substrate interactions. J. Virol. *66*, 4315–4324.

Lim, M.-Y., Dailey, D., Martin, G.S., and Thorner, J. (1993). Yeast *MCK1* protein kinase autophosphorylates at tyrosine and serine but phosphorylates exogenous substrates at serine and threonine. J. Biol. Chem. *268*, 21155–21164.

Lindberg, R.A., Quinn, A.M., and Hunter, T. (1992). Dual-specificity protein kinases: will any hydroxyl do? Trends Biol. Sci. *17*, 114–119.

Litwin, C.M., Cheng, H.C., and Wang, J.H. (1991). Purification and characterization of a $pp60^{c\text{-}src}$-related tyrosine kinase that effectively phosphorylates a synthetic peptide derived from p34cdc2. J. Biol. Chem. *266*, 2557–2566.

Maclennan, A.J., and Shaw, G. (1993). A yeast SH2 domain. Trends Biochem. Sci. *18*, 464–465.

McGowan, C.H., and Russell, P. (1993). Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. EMBO J. *12*, 75–85.

Millar, J.B.A., Lenaers, G., and Russell, P. (1992). *Pyp3* PTPase acts as a mitotic inducer in fission yeast. EMBO J. *11*, 4933–4941.

Muller, A.J., Pendergast, A.M., Havlik, M.H., Puil, L., Pawson, T., and Witte, O.N. (1992). A limited set of SH2 domains binds BCR through a high-affinity phosphotyrosine-independent interaction. Mol. Cell. Biol. *12*, 5087–5093.

Murphy, S.M., Bergman, M., and Morgan, D.O. (1993). Suppression of c-Src activity by C-terminal Src kinase involves the c-Src SH2 and SH3 domains: analysis with *Saccharomyces cerevisiae*. Mol. Cell. Biol. *13*, 5290–5300.

Murray, A.W. (1992). Cell-cycle checkpoints and feedback controls. Nature *359*, 599–604.

Obar, R.A., Collins, C.A., Hammarback, J.A., Shpetner, H.S., and Vallee, R.B. (1990). Molecular cloning of the microtubule-associated mechanochemical enzyme dynamin reveals homology with a new family of GTP-binding proteins. Nature *347*, 256–261.

Ottilie, S., Chernoff, J., Hannig, G., Hoffman, C.S., and Erikson, R.L. (1992). The fission yeast genes $pyp1^+$ and $pyp2^+$ encode protein tyrosine phosphatases that negatively regulate mitosis. Mol. Cell. Biol. *12*, 5571–5580.

Parsons, J.T., and Weber, M.J. (1989). Genetics of src: structure and functional organization of a protein tyrosine kinase. Curr. Top. Microbiol. Immunol. *147*, 79–127.

Pawson, T., and Gish, G.D. (1992). SH2 and SH3 domains: from structure to function. Cell *71*, 359–362.

Pawson, T., and Schlessinger, J. (1993). SH2 and SH3 domains. Curr. Biol. *3*, 434–442.

Pazin, M.J., and Williams, L.T. (1992). Triggering signaling cascades by receptor tyrosine kinases. Trends Biochem. Sci. *17*, 374–378.

Ren, R., Mayer, B.J., Cicchetti, P., and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. Science *259*, 1157–1161.

Rodaway, A.R., Teahan, C.G., Casimir, C.M., Segal, A.W., and Bentley, D.L. (1990). Characterization of the 47-kilodalton autosomal chronic granulomatous disease protein: tissue-specific expression and transcriptional control by retinoic acid. Mol. Cell. Biol. *10*, 5388–5396.

Russell, P., Moreno, S., and Reed, S.I. (1989). Conservation of mitotic controls in fission and budding yeasts. Cell *57*, 295–303.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genet. *122*, 19–27.

Solomon, M.J., Harper, J.W., and Shuttleworth, J. (1993). CAK, the p34cdc2 activating kinase, contains a protein identical or closely related to p40MO15. EMBO J. *12*, 3133–3142.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., Neel, B.G., Birge, R.B., Fajardo, J.E., Chou, M.M., Hanafusa, H., Schaffhausen, B., and Cantley, L.C. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767–778.

Sorger, P.K., and Murray, A.W. (1992). S-phase feedback control in budding yeast independent of tyrosine phosphorylation of $p34^{cdc28}$. Nature *355*, 365–368.

Stern, D., Zheng, P., Beidler, D.R., and Zerillo, C. (1991). Spk1, a new kinase from *Saccharomyces cerevisiae*, phosphorylates proteins on serine, threonine and tyrosine. Mol. Cell. Biol. *11*, 987–1001.

Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S.A., and Draetta, G. (1993). Csk inhibition of c-Src kinase involves the c-Src SH2 and SH3 domains of Src. EMBO J. *12*, 2625–2634.

Tarpley, W.G., and Temin, H.M. (1984). The location of v-*src* in a retrovirus vector determines whether the virus is toxic or transforming. Mol. Cell. Biol. *4*, 2653–2660.

Tonks, N.K., Charbonneau, H., Diltz, C.D., Kumar, S., Cicirelli, M.F., Krebs, E.G., Walsh, K.A., and Fischer, E.H. (1989). Protein tyrosine phosphatases: structure, properties and role in signal transduction. Adv. Prot. Phosphatases *5*, 149–180.

Wan, J., Xu, H., and Grunstein, M. (1992). CDC14 of *Saccharomyces cerevisiae*. Cloning, sequence analysis, and transcription during the cell cycle. J. Biol. Chem. *267*, 11274–11280.

Wang, E., and Goldberg, A.R. (1979). Effects of the src gene product on microfilament and microtubule organization in avian and mammalian cells infected with the same temperature-sensitive mutant of Rous sarcoma virus. Virol. *92*, 201–210.

Wasenius, V.M., Saraste, M., and Lehto, V.P. (1989). From the spectrin gene to the assembly of the membrane skeleton. Int. J. Dev. Biol. *33*, 49–54.

Wilson, I.A., Niman, H.L., Houghten, R.A., Cherenson, A.R., Connolly, M.L., and Lerner, R.A. (1984). The structure of an antigenic determinant in a protein. Cell *37*, 767–778.

Woodford-Thomas, T.A., Rhodes, J.D., and Dixon, J.E. (1992). Expression of a protein tyrosine phosphatase in normal and v-*src*-transformed mouse 3T3 fibroblasts. J. Cell Biol. *117*, 401–414.

Xu, Y., and Lindquist, S. (1993). Heat-shock protein hsp90 governs the activity of $pp60^{v\text{-}src}$ kinase. Proc. Natl. Acad. Sci. USA *90*, 7074–7078.

Zheng, P., Fay, D.S., Burton, J., Xiao, H., Pinkham, J.L., and Stern, D.F. (1993). SPK1 is an essential S-phase-specific gene of *Saccharomyces crevisiae* that encodes a nuclear serine/threonine/tyrosine kinase. Mol. Cell. Biol. *13*, 5829–5842.