Analysis of the interaction between piD261/Bud32, an evolutionarily conserved protein kinase of *Saccharomyces cerevisiae*, and the Grx4 glutaredoxin

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The *Saccharomyces cerevisiae* piD261/Bud32 protein and its structural homologues, which are present along the Archaea–Eukarya lineage, constitute a novel protein kinase family (the piD261 family) distantly related in sequence to the eukaryotic protein kinase superfamily. It has been demonstrated that the yeast protein displays Ser/Thr phosphotransferase activity *in vitro* and contains all the invariant residues of the family. This novel protein kinase appears to play an important cellular role as deletion in yeast of the gene encoding piD261/Bud32 results in the alteration of fundamental processes such as cell growth and sporulation. In this work we show that the phosphotransferase activity of Bud32 is relevant to its functionality *in vivo*, but is not the unique role of the protein, since mutants which have lost catalytic activity but not native conformation can partially complement the disruption of the gene encoding piD261/Bud32. A two-hybrid approach has led to the identification of several proteins interacting with Bud32; in particular a glutaredoxin (Grx4), a putative glycoprotease (Ykr038/Kae1) and proteins of the Imd (inosine monophosphate dehydrogenase) family seem most plausible interactors. We further demonstrate that Grx4 directly interacts with Bud32 and that it is phosphorylated *in vitro* by Bud32 at Ser-134. The functional significance of the interaction between Bud32 and the putative protease Ykr038/Kae1 is supported by its evolutionary conservation.

Key words: glutaredoxin, glycoprotease, protein kinase, protein–protein interaction, yeast.

INTRODUCTION

Sequence analysis of the genomes of several prokaryotic and eukaryotic organisms has disclosed a number of putative protein kinase families that are distantly related in sequence to the eukaryotic protein serine/threonine kinase superfamily and that are present in both prokaryotes and eukaryotes [1]. To one of these, the piD261 family, belongs the product of the *Saccharomyces cerevisiae YGR262c/BUD32* gene, piD261/Bud32, [2] and its structural homologues, that are present in virtually all eukaryotes and in archaebacteria, but not in eubacteria.

The relevant features of the piD261/Bud32 protein are the small size (261 residues), its low similarity with the other members of the eukaryotic protein kinase family and the alteration of some of the motifs highly conserved in the family.

Despite these structural abnormalities piD261/Bud32, expressed in *Escherichia coli* as a recombinant protein, acts *in vitro* as a Ser/Thr protein kinase, recognizing acidic substrates and depending on Mn^{2+}, instead of Mg^{2+}, for catalysis [3,4]. A recent mutational analysis has demonstrated that, despite the low overall similarity, the invariant residues representing the signature of protein kinases are in fact conserved in piD261/Bud32, but are embedded in an altered context, suggestive of unique mechanistic properties [5]. All the main structural features of piD261/Bud32 are conserved in the homologous proteins present in higher eukaryotes, notably the human one, suggesting that they underlie common biological function(s) in distantly related organisms.

The biological functions of piD261/Bud32 and of the other members of the family are unknown; they must be crucial for key cellular processes, however, since deletion of the yeast *YGR262c/BUD32* gene dramatically inhibits mitotic growth, resulting in a severe slow-growth phenotype [6]. Homozygous diploid mutants are unable to enter sporulation, with no visible meiotic division [7]. Accordingly, results of yeast transcriptome analysis [8] indicate an enhancement of *YGR262c/BUD32* transcription during sporulation. Mutant cells also display morphogenetic defects, notably alterations in cell-wall structure [9] and budding [10]. Due to the latter phenotype the name *BUD32* has been attributed to the *YGR262c* gene.

The human structural homologue of Bud32, named PRPK (p53-related protein kinase), has proved to be a functional homologue of the yeast protein, as it partially complements the phenotype resulting from deletion of the yeast *YGR262c/BUD32* gene [11]. PRPK must also be implicated in cell proliferation, as it is able to bind to p53 and to phosphorylate it at Ser-15, a key event for cell-cycle arrest due to genotoxic conditions [12]. Interestingly, PRPK mRNA is markedly expressed, among normal human tissues, only in testis, while its expression is considerably elevated in interleukin-2-activated cytotoxic T-cells and in some human epithelial tumour cell lines [12].

These observations prompted us to start an investigation aimed at assessing the functional relevance of Bud32 phosphotransferase activity in the cell and disclosing its targets and partners in yeast. Here we show that the phosphotransferase activity of the Bud32

Abbreviations used: PRPK, p53-related protein kinase; IMD, inosine monophosphate dehydrogenase; Grx, glutaredoxin; PKA, cAMP-dependent protein kinase; GFP, green fluorescent protein; Gal4 BD, Gal4 DNA-binding domain; Gal4 AD, Gal4 transcriptional activation domain; SPR, surface plasmon resonance; HA, haemagglutinin; MALDI, matrix-assisted laser-desorption ionization.

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protein is relevant to its functionality *in vivo*, but also that the protein must have a more complex role in the cell. By the two-hybrid approach we have identified several Bud32-interacting proteins, among which a putative glycoprotease, Ykr038 the Grx4 glutaredoxin and proteins encoded by the *IMD* (inosine monophosphate dehydrogenase) gene family appear to be most probable interactors. The demonstration that Grx4 is phosphorylated by Bud32 *in vitro* strongly suggests that the cellular activity of Grx4 might be modulated by the Bud32 protein kinase.

EXPERIMENTAL

Strains and media

Yeast strains were as follows: W303-1B (*Mat* **a**; *ade2-1*; *his3-11*,*15*; *leu2-3*,*112*; *trp1-1*; *ura3-1*; *can*^{R}); W303-1B +/Δ*BUD32* (**a**/**α**, *ade2-1*/*ade2-1*, *his3-11*,*15*/*his3-11*,*15*, *leu2-3*,*112*/*leu2-3*,*112*, *trp1-1*/*trp1-1*, *ura3-1*/*ura3-1*, *can1-100*/*can1-100*; *BUD32*/*bud32*::*kanMX4*); W303-1B Δ*BUD32* (*Mat* **a**; *ade2-1*; *his3-11*,*15*; *leu2-3*,*112*; *trp1-1*; *ura3-1*; *can*^{R}; *bud32*::*kanMX4*); and Y190 (*Mat* **a**; *ura3-52*; *his3-200*; *ade2-101*; *lys2-801*; *trp1-901*; *leu2-3*,*112*; *gal4*Δ; *gal80*Δ; *cyh*^{r}*2*; *LYS2*::*GAL1*$_{UAS}$-*HIS3*$_{TATA}$-*HIS3*; *URA3*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*).

E. coli strains were as follows: Invα F′ [*endA1, recA1, hsdR17* (r^{-K}, m^{-K}), *supE44,* λ^{-}, *thi-1, gyrA, relA1,* Φ*80lacZ*αΔ*M15*Δ (*lacZYA-argF*), *deoR*$^{+}$, *F*′]; MC1066 [*pyrF74*::*Tn5* (*Km*^{r}) *leuB6 ara*$^{+}$ *trpC9830* Δ(*ara leu*), Δ(*lacIPOZYA*)*X74 galU galK StrA*^{r}]; and BL21(DE3) [*F*$^{-}$ *ompT hsdS*$_{B}$ (r_{B}^{-} m_{B}^{-}) *gal dcm* (*DE3*)].

The following media were used: YPD (1 % yeast extract/1 % bactopeptone/2 % dextrose); SD (0.67 % yeast nitrogen base without amino acids/2 % dextrose/auxotrophic requirements as needed); adenine-limiting (SD medium with 10 mg/l adenine); sporulation (1 % potassium acetate); and Luria–Bertani (1 % Bactotryptone/0.5 % yeast extract/0.5 % NaCl; when requested, ampicillin was added to a final concentration of 100 μg/ml). Solid media were obtained by the addition of 2 % agar. Media components were from Difco, and amino acids were from Sigma.

Phenotype-complementation assay

The pFL-261 plasmid contains the *BUD32* coding sequence under the control of the *BUD32* promoter. The sequence has been obtained by amplification of the *S. cerevisiae* chromosomal region comprising the *BUD32* coding sequence surrounded by 723 bp upstream and 189 bp downstream, using the primers 5′-PROM (5′-CTGCTGGATCCAATGCTGTTTTG-3′) and 3′-PROM (5′-GTTTGCTGCAGTTGGTTTACC-3′), which introduce the restriction sites *Bam*HI and *Pst*I respectively (underlined). The resulting amplification product was cloned into the centromeric pFL-38 vector [13] at the *Bam*HI/*Pst*I sites.

The same insert was also cloned into the *Bam*HI/*Pst*I sites of plasmid pASZ11 [14] to obtain the *BUD32*-pASZ11 vector. This is a yeast centromeric plasmid carrying the *ADE2* gene as a selectable marker. After transformation of an *ade2* mutant strain, the colonies formed by yeast cells containing the plasmid are white and can be isolated by growth on selective medium lacking adenine. Subsequent growth of these cells on an adenine-limiting medium determines a spontaneous loss of plasmid that can be easily monitored by the colour change of the colonies, which become red. The use of this plasmid is particularly useful because it allows the maintenance of an extra-chromosomal copy of one gene in the yeast cell, but also, when necessary, it allows us to obtain the loss of this gene copy by a simple shift in the appropriate medium followed by a colour-monitoring of the growing colonies. This plasmid was used to transform a +/Δ*BUD32* heterozygous diploid yeast strain and, after tetrad dissection of transformed diploids, to isolate Δ*BUD32* haploid cells carrying the extra-chromosomal copy of the *BUD32* gene, giving the 'protected' W303-1B Δ*BUD32* haploid yeast strain used for the complementation assays.

Mutagenesis of the *BUD32* gene

The mutant forms of the *BUD32* gene were obtained using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene; catalogue no. 200518). The mutagenic primers were designed according to the instruction manual. As double-stranded DNA templates we used the plasmidic construct pFL-261 described above.

Tetrad dissection

The W303-1B +/Δ*BUD32* heterozygous diploids transformed with the pFL-261 plasmid (or the pFL-261 plasmid carrying a mutated *BUD32* coding sequence) were allowed to sporulate on 1 % potassium acetate solid medium for 4–6 days at 25 °C. About 15 tetrads/diploid transformant strain were dissected and the spores allowed to germinate on YPD medium at 28 °C. For each spore, the geneticin-resistant phenotype (indicating the presence of the Δ*BUD32* allele) and the presence of the pFL-261 plasmid were controlled by growth on selective media.

Two-hybrid screening

The entire 783 bp *BUD32* open reading frame was amplified by PCR from yeast genomic DNA using appropriate primers, 5′-BAIT (5′-CGCTTTGGATCCTTATGACGCAAGAA-3′) and 3′-BAIT (5′-GGAATTCCTGCAGGGTACCC-3′), containing the *Bam*HI and *Pst*I recognition sequences respectively (underlined). The resulting amplified fragment, digested with *Bam*HI and *Pst*I, was cloned in frame to the Gal4 BD (Gal4 DNA-binding domain) coding sequence in vector pGBT9 (Clontech), previously digested with the same two enzymes. The resulting plasmid, pGBT9-Bud32, encodes the fusion protein Gal4 BD–Bud32. This retains the physiological function of Bud32, as the yeast Δ*BUD32* strain transformed with the pGBT9-Bud32 plasmid showed complete recovery of the wild-type phenotype. The bait pGBT9-Bud32 plasmid was used to transform the reporter *S. cerevisiae* strain Y190 (Clontech). The resulting strain was transformed using a high-efficiency lithium acetate transformation method [15], with a yeast chromosomal DNA library fused to the Gal4 AD (Gal4 transcriptional activation domain) and to the influenza HA (haemagglutinin) epitope. The library (FRYL) was kindly provided by M. Fromont-Racine and P. Legrain (Institut Pasteur, Paris, France) [16]. Transformants containing both bait and library plasmids and able to activate transcription of the *HIS3* reporter gene were selected for growth on selective minimal medium lacking leucine, tryptophan, histidine and containing 60 mM 3-AT (3-amino-1,2,4-triazole; Sigma). Next, these first-screening positive clones were tested for the activation of the second reporter gene (*lacZ*) by their ability to generate blue colonies in the presence of X-gal. Library plasmids were extracted from positive yeast clones and separated from bait plasmids by transformation of the MC1066 *E. coli* strain [17]. These candidate plasmids were reintroduced into the Y190 yeast strain together with the original bait plasmid pGBT9-Bud32, or the pGBT9 plasmid alone, in order to confirm the interaction. The inserts of the plasmids that overcame these selective steps were sequenced and their identity

determined by database searching: the exact position of the fusion with *GAL4* was identified for each prey by sequencing the 5′-end of the insert; sequencing of the 3′-end was necessary only for inserts longer than 700–800 bp.

Expression of His-tagged recombinant proteins

The plasmid pET-261 [3] was used to express the C-terminal His_6-tagged Bud32 recombinant protein in *E. coli* cells. The protocol steps for protein purification are described elsewhere [5].

The coding sequence of the *GRX4* gene was amplified by PCR from yeast genomic DNA using primers 5′-GRX4 (5′-TCCCGGGTCATATGTTATGACTGTGG-3′) and 3′-GRX4 (5′-AGGCTCGAGCTGTAGAGCATGTTGG-3′) that introduced the restriction sites for *Nde*I and *Xho*I (underlined): the PCR product was cloned into the pET-20b(+) plasmid (Novagen) digested with the same enzymes, giving the pET-Grx4 expression vector. For the purification of Grx4–His_6, the *E. coli* strain BL21(DE3), transformed with plasmid pET-Grx4, was grown in Luria–Bertani medium at 37 °C until the D_{600} value reached 0.7–0.8, when transcription of the *GRX4* coding sequence was induced with 0.4 mM isopropyl β-D-thiogalactoside (Calbiochem). After 16–20 h of incubation at 16 °C bacteria were harvested and resuspended in 5 ml/g of pellet of purification buffer (0.02 M Tris/HCl, pH 7.5, 0.3 M NaCl, 10 % glycerol and 0.2 mM PMSF) in the presence of a protease inhibitor cocktail (Boehringer Mannheim). Purification was performed according to the protocol of the manufacturer by an affinity column containing Ni^{2+}-nitrilotriacetate–agarose (Qiagen). The 27 kDa Grx4–His_6 was eluted with 0.04 M imidazole. All protein concentrations were determined by the method of Bradford [18] using BSA as a standard.

Immunoprecipitation and immunoblotting

Yeast cells transformed with the appropriate plasmid were grown in 100 ml of selective medium until mid-log phase (approx. 10^7 cells/ml) and cells were harvested by centrifugation. The cells were lysed by vortexing for 7 min on ice in breaking buffer [1:1, w/v; 0.05 M Hepes/0.3 M NaCl/20 % glycerol/0.001 M sodium orthovanadate/0.06 M β-glycerophosphate/Complete anti-protease cocktail (Boehringer Mannheim)] in the presence of an identical volume of glass beads. The insoluble fraction was removed by centrifugation (20 min at 10 000 ***g*** at 4 °C) and the protein concentration of the soluble fraction was determined by the Bradford method. The soluble fraction was divided into aliquots of 6 mg of total proteins and a pre-cleaning of the solution was performed with Protein-A–Sepharose (Sigma), saturated with BSA and equilibrated with breaking buffer, to reduce non-specific binding. Samples were then incubated with anti-HA antibodies (Boehringer Mannheim) at 4 °C for 2 h. The immunocomplexes were precipitated with Protein-A–Sepharose; the pellets were washed three times with PBS, blocked in a buffer consisting of 0.6 M Tris/HCl, pH 6.8, 2 % SDS, 20 % glycerol and 0.005 M β-mercaptoethanol and subjected to SDS/PAGE (11 % gels). The proteins were blotted on to PVDF membrane (Millipore) and detected using mouse antiserum against Bud32–His_6 as described in [5], or monoclonal anti-HA antibodies (Boehringer Mannheim).

SPR (surface plasmon resonance) analysis

For the SPR analysis, a BIAcoreX system was used, as described in [19], with the following modifications. Recombinant Bud32 was covalently immobilized on the surface of a flow cell in a CM5 sensor chip, to a final density of 2600 resonance units, while a flow cell with no immobilized protein was used as a control. The interacting protein solutions of Grx4 were injected at a flow rate of 10 μl/min, and each sensorgram (time course of the SPR signal) was corrected for the response obtained in the control flow cell. Surface was regenerated by injection of 0.01 M NaOH, with a contact time of 1 min. The kinetic data were interpreted with a simple 1:1 binding model, and the rate constants of the interactions were calculated by using the BIAevaluation 3.0 SPR kinetic software (BIAcore). The reported values are the means from three separate experiments, with S.E.M. values lower than 10 %.

Phosphotransferase assay

The protein kinase activity of Bud32 was assayed as described elsewhere [5] by incubating the recombinant His-tagged protein (0.3 μM final concentration) at 37 °C for 15 min in 20 μl of medium containing 0.05 M Tris/HCl, pH 7.5, 0.01 M $MnCl_2$ and 25 μM [γ-^{32}P]ATP (Amersham Biosciences; specific radioactivity, 2000–3000 c.p.m./pmol) and the Grx4 protein (0.9–1.8 μM final concentration) as phosphorylatable substrate. The reaction was stopped by adding the gel electrophoresis loading buffer and samples were subjected to SDS/PAGE (11 % gels). The dried gels were scanned directly using the Cyclone apparatus (Packard).

Fluorescence analysis

In order to determine the subcellular localization of both the Bud32 and Grx4 proteins, we expressed them as fusion proteins to the C-terminus of the GFP (green fluorescent protein). The coding sequence of the *BUD32* and *GRX4* genes were amplified from yeast genomic DNA by PCR using the primers 5′-GFP-BUD32 (5′-CCGGATCCATGACGCAAGAATTCATTG-3′) and 3′-GFP-BUD32 (5′-ACTCGAGTTATCCTAGCATACTTCTCTTAC-3′) for the *BUD32* gene and 5′-GFP-GRX4 (5′-CGGATCCATGATGAGTTCGAGAGTAACC-3′) and 3′-GFP-GRX4 (5′-GCTCGAGATTACTGTAGAGCATGTTG-3′) for the *GRX4* gene, carrying the restriction sites for *Bam*HI and *Xho*I (underlined): the amplification products were cloned into the pUG-36 plasmid [20], digested with the same enzymes, downstream of the GFP coding sequence. The conservation of the correct reading frame of the fusion was verified by sequencing. To ascertain whether the GFP–Bud32 fusion protein maintains its functionality, yeast cells carrying the inactivation of the *BUD32* gene were transformed with the Bud32-pUG-36 plasmid and it could be observed that the fusion protein fully complemented the slow-growth phenotype. Yeast cells were transformed with Bud32-pUG-36 or Grx4-pUG-36 and the fluorescence analysis was performed using an Olympus IMT-2 microscope. Fluorescence analysis and preparation of samples were performed according to [20]. Image analysis was performed using Metamorph software (Universal Imaging, Downingtown, PA, U.S.A.).

MS analysis

MALDI (matrix-assisted laser-desorption ionization) analysis was carried out using a MALDI-Tof (Micromass) on the tryptic digest before and after treating the sample with $Ba(OH)_2$. The reaction with $Ba(OH)_2$ implies a β-elimination of H_3PO_4 and the generation of dehydrated serine (or threonine) at the site of phosphorylation [21–23].

Tryptic digestion of Grx4

The gel bands containing phosphorylated Grx4 were excised and subjected to reduction, alkylation and tryptic digestion according to [24]. Briefly, Coomassie-stained bands were excised, dehydrated with acetonitrile, reduced with 0.01 M dithiothreitol for 1 h at 56 °C and then carboxyamidomethylated with 0.055 M iodacetamide in the dark for 45 min. The gel pieces were dehydrated with acetonitrile and rehydrated with 0.05 M ammonium bicarbonate containing 12.5 ng/μl trypsin. After overnight digestion at 37 °C, the peptides were extracted by washing the gel pieces three times with 50 % acetonitrile/5 % formic acid. The solution was concentrated in Speed Vac and then desalted using a C_{18} Zip-Tip (Millipore).

Chemical dephosphorylation of the peptides

Digested protein (1 μl) was mixed with 1 μl of matrix [5 mg/ml α-cyano-4-hydroxycinnamic acid (Aldrich) in 50 % acetonitrile/0.1 % formic acid] and spotted on to the target plate. The sample was treated with a saturated solution of $Ba(OH)_2$ for 20 min and was desalted using a C_{18} Zip-Tip (Millipore), mixed with the matrix and spotted on to the target plate. The spectra were inspected manually and compared to identify probable phosphopeptides subjected to chemical dephosphorylation. The identification of the phosphorylation sites was made by acquiring MS/MS spectra of the chemically dephosphorylated peptides using an Ultima Q-Tof (Micromass). The spectra were interpreted manually according to [25] and the phosphorylation sites identified unequivocally.

RESULTS AND DISCUSSION

Cellular function of Bud32 does not exclusively rely on its phosphotransferase activity

Deletion in *S. cerevisiae* of the *YGR262c*/*BUD32* gene, encoding piD261/Bud32, confers to mutant strains a pleiotropic phenotype resulting in a very slow vegetative growth of colonies. This phenotype is complemented by ectopic expression of the wild-type *BUD32* sequence [6]. To establish whether and to what extent the functionality of the Bud32 protein *in vivo* relies on its phosphotransferase activity, the complementation of the slow-growth phenotype by the Bud32 wild-type protein was compared with that obtained with mutants shown previously *in vitro* to be severely defective in catalytic activity [5]. These are mutants G25V (in which the only residual Gly of the glycine loop is substituted by Val), K52A [in which the lysine corresponding to K72 of PKA (cAMP-dependent protein kinase) is substituted by Ala] and D161A (in which the aspartic acid corresponding to the catalytic base, Asp-166, of PKA is substituted by an Ala residue). As shown in Figure 1, the three mutated *BUD32* sequences, inserted in a centromeric plasmid under the control of the *BUD32* gene promoter, were not able to fully restore the wild-type growth phenotype of Δ*BUD32* cells as did the wild-type *BUD32* sequence inserted in the same plasmid. In particular the effect is observed on the growth of spores issued from the dissection of a heterozygous diploid +/Δ*BUD32* transformed with the different plasmids (Figure 1, top panels; compare the sizes of colonies formed by Δ*BUD32* spores in the presence of the mutant alleles and those of Δ*BUD32* spores in the presence of the wild-type allele). However, when the same segregants are replated, their colonies grow at rates very similar to the wild-type. Accordingly, when haploid vegetative Δ*BUD32* cells are transformed with plasmids carrying the mutant alleles, complementation of the phenotype is lower than that observed with the wild-type allele, but the difference is slight (Figure 1, lower panel). In this case, Δ*BUD32* cells were 'protected' and the phenotype due to complementation was observed after loss of

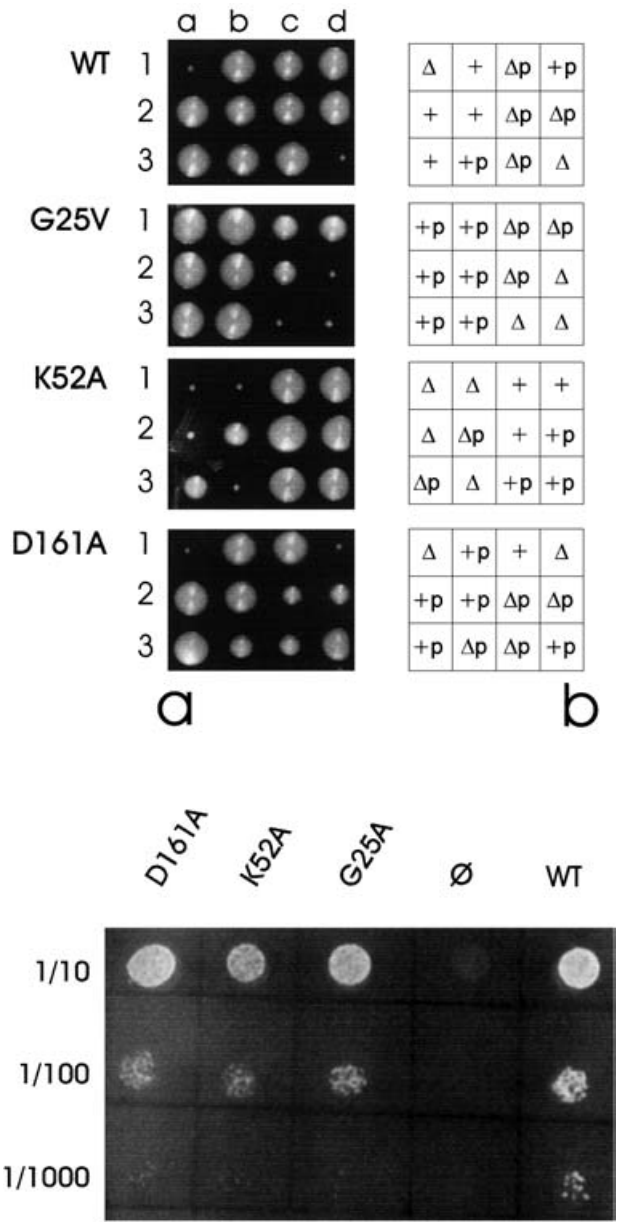

Figure 1 Mutant forms of the *BUD32* gene partially complement the W303-Δ*BUD32* phenotype

Top panels: complementation assay on germination after sporulation. Cells of the heterozygous W303-1B +/Δ*BUD32* diploid strain were transformed with the pFL-261 plasmid (WT) or with the pFL-261 plasmid carrying a mutated *BUD32* coding sequence (notably G25V, K52A, D161A). Transformants were allowed to sporulate and tetrads dissected. The resulting spores were grown at 28 °C and observed after 4 days of incubation. For each diploid transformed strain three tetrads are shown (**a**). (**b**) The segregation of the wild-type genomic *BUD32* allele (+) and of the deleted form (Δ) of it is indicated. The letter p indicates the presence of the pFL-261 plasmid (WT or mutated). Bottom panel: complementation assay on vegetative growth. Cells from the 'protected' W303–1B Δ*BUD32* haploid strain (see the Experimental section) were transformed with the pFL-38 plasmid without insert (Ø), the pFL-261 plasmid (WT) or with the pFL-261 plasmid carrying the same mutated *BUD32* coding sequences indicated in the top panels. After loss of the *BUD32*-pASZ11 plasmid, each type of transformant was grown in liquid selective medium until stationary phase, diluted at 3×10^7 cells/ml and spotted on to solid medium in serial dilution (1/10, 1/100, 1/1000). Growth was observed after 2 days at 28 °C.

Table 1 Bud32-interacting proteins identified by two-hybrid screening

Description and Function columns give information that is available on the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). ORF, open reading frame; aa, amino acid.

Clone	Gene/ORF	Protein length (aa)	Region of protein found (aa)	Description	Function (assigned or putative)
1	*GRX4**	245	103–STOP	Grx	Response to oxidative stress
2			148–STOP		
3			55–STOP		
4			99–STOP		
5			102–STOP		
6			12–STOP		
7			1–STOP		
8			105–STOP		
9			105–STOP		
10	*GRX3**	286	96–260	Grx	Response to oxidative stress
11	*YKR038c**	421	134–368	Similarity with O-sialoglycoprotease	Unknown
12			168–STOP		
13			180–STOP		
14			163–346		
15	*IMD1*	404	104–262	IMP dehydrogenase homologue	Nucleotide metabolism
16			167–235		
17	*IMD2**	524	205–330	IMP dehydrogenase homologue	Nucleotide metabolism
18			205–330		
19	*IMD4**	525	154–260	IMP dehydrogenase homologue	Nucleotide metabolism
20	*XPO1* *CRM1**	1085	943–STOP	Exportin 1	Involved in nuclear export
21	*VAC8*	579	200–STOP	Protein of the vacuolar membrane	Protein-vacuolar targeting
22	*MET10*	1035	504–638	Sulphite reductase	Sulphate assimilation
23	*TRK1*	1236	71–348	Potassium transporter	Potassium ion homoeostasis
24	*KRE33*	1057	681–1045	Killer toxin resistant	Unknown

* Proteins also identified in the complex characterized by Ho et al. [38].

the *BUD32*-pASZ11 plasmid (see the Experimental section). This result suggests that mutations in Bud32 residues that have an essential role in the phosphotransferase reaction, but are not expected to alter the overall conformation of the protein, mainly cause a defect in the spore germination processes. This effect has been observed only in the case of 'catalytic' mutations in the Bud32 protein kinase, while other Bud32 mutations (e.g. K57A), which do not alter its *in vitro* catalytic activity [5], behave as the wild-type. Since post-germination growth defects have been observed in several cases of mutations in genes involved in the biogenesis and integrity of the cell wall [26–28], and *YGR262c*/*BUD32* has been classified as a cell-wall-related gene [9], it is possible that the result is due to a difficulty of the mutants to modify the spore wall, a process necessary to permit spore outgrowth and formation of the initial bud.

These results allow us to establish a correlation between the biological competence of Bud32 and its phosphotransferase activity. However, they also show that the *presence* of Bud32, even in a catalytically inactive form, is able to significantly rescue the severe phenotype of the Δ*BUD32* mutant. Without completely excluding the possibility that, in the presence of the cellular context, the partial complementation of the phenotype is due to a more than physiological concentration of the mutant proteins (owing to the potential presence of more than one single copy of the centromeric plasmid) resulting in an overall significant, albeit low, catalytic activity, this result suggests that, in addition to its protein kinase activity, the Bud32 protein might have other cellular roles. One possibility would be that the presence of the Bud32 protein, whether devoid of catalytic activity or not, allows the establishment of functional interactions with other proteins, whose activity is normally regulated by the Bud32 protein, leading to a partial suppression of the Δ*BUD32* phenotype. This prompted us to search the yeast genome for genes expressing proteins able to interact with Bud32.

Identification of cellular partners of Bud32 by the Gal4-based two-hybrid system

Using Bud32 as a 'bait' (Gal4 BD–Bud32) we have screened a yeast genomic expression library to search for polypeptides (Gal4 AD-Preys) able to interact with Bud32. We obtained approx. 3×10^6 independent transformants, 89 of which were positive to the first reporter gene *HIS3*. Of these, 24 clones were positive also to the second reporter gene, *lacZ*. After confirmation of the interaction with Bud32 for all the polypeptides expressed by the positive plasmids, the inserts were identified by DNA sequencing. As it can be seen in Table 1, the inserts of the isolated 24 clones represent different fragments of the yeast genome that identify 11 different genes. In several cases, only one fragment represents a given gene, while four genes (*GRX4*, *YKR038c*, *IMD1*, *IMD2*) are identified by more than one fragment. In addition, two clones contain fragments of genes *GRX3* and *IMD4*, which are highly homologous respectively to *GRX4* and *IMD1*/*IMD2*. On the basis of the number of different fusions recovered from the same prey protein we conclude that Grx4, Ykr038 and proteins encoded by the IMD gene family are the most plausible interactors of Bud32. We focused our attention on Grx4, which has been identified unambiguously by several clones (9/24 clones), and tried to confirm its interaction with Bud32 using alternative approaches.

However, the interactions of Bud32 with Ykr038 and the Imd proteins also seem interesting and merit further study. IMP dehydrogenases are the rate-limiting enzymes in the *de novo* synthesis of guanine nucleotides and appear to play a crucial role in the growth of many cell types (see [29] for a review), including yeast [30]. The putative association with these proteins might be related to the proposed involvement of Bud32 in the control of cellular proliferation, indicated by the slow-growth phenotype due to *YGR262*/*BUD32* deletion [6]. As regards Ykr038, though a functional characterization of the protein is lacking, its significant

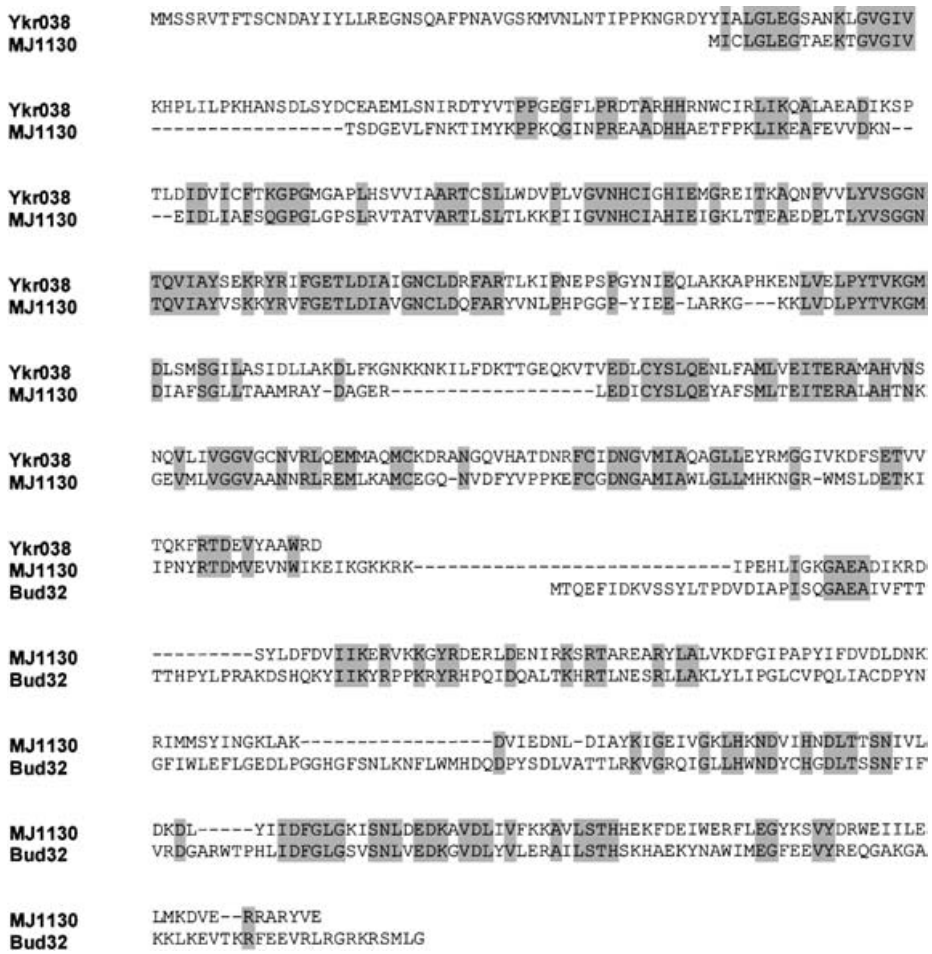

Figure 2 Structure of the *Methanococcus jannaschii* putative glycoprotease (MJ1130)

The MJ1130 protein (Swissprot accession no. Q58530) can be subdivided into two regions: a N-terminus similar to the yeast Ykr038 protein and a C-terminus similar to the Bud32 protein. The identical residues are indicated on grey backgrounds.

sequence similarity with a O-sialo-glycoprotease characterized from *Pasteurella haemolytica* [31] suggests that the protein, whose primary structure appears to be conserved all along the evolutionary timescale, might be endowed with proteolytic activity. In *S. cerevisiae* the integrity of the *YKR038c* gene is essential for cell life, as its inactivation is lethal ([32], and R. Lopreiato and G. Sartori, unpublished work). The functional significance of the Bud32–Ykr038 association, identified by our two-hybrid system, is highlighted by the observation that in some species of *Methanococcus* (Archaea), a single gene encodes a polypeptide formed by an N-terminal region similar to the Ykr038 protein and a C-terminal region similar to the Bud32 protein kinase (Figure 2). Thus the interaction observed in yeast between the two proteins suggests an evolutionary mechanism of separation of the genes and conservation of the functional interaction between the two gene products. In accordance with these observations, we have reserved at *Saccharomyces* Genome Database (http://www.yeastgenome.org/) the name *KAE1* (kinase-associated endopeptidase 1) for the *YKR038c* gene.

Grx4 interacts directly with Bud32 and is phosphorylated by it at Ser-134

The Grx4 protein is a member of the Grx superfamily, involved in maintaining the redox state of cellular proteins and comprising mainly thiol-disulphide oxidoreductases. Grx4 belongs to a subfamily of yeast Grxs (Grx3, Grx4 and Grx5) that contain a single cysteine residue at the active site. This trait is also characteristic of a number of Grxs from bacteria to humans, with which the Grx3/4/5 group shares extensive homology over two regions. While the role of Grx5 has been in part clarified, no evidence yet has been provided for the specific roles of Grx3 and Grx4 [33,34]. Thiol-disulphide oxidoreductases have often been found associated with protein kinases and phosphatases in mammals, where they are believed to regulate the activity of these enzymes in response to redox signals [35–37].

Bud32 and Grx4 interact directly

The interaction between Bud32 and Grx4 was confirmed by a number of different techniques. The first was by a reciprocal two-hybrid assay, which showed that reconstitution of the Gal4 activator occurs also when the Grx4 protein is fused to Gal4 BD and the Bud32 protein to Gal4 AD.

The second technique was by co-immunoprecipitation, taking advantage of wild-type yeast cells transformed with one of the Bud32-interacting plasmids that express a fusion protein Gal4 AD–HA epitope–Grx4, which was then immunoprecipitated from cell lysates with commercial anti-HA antibodies. After analysis by SDS/PAGE and Western blot it could be observed

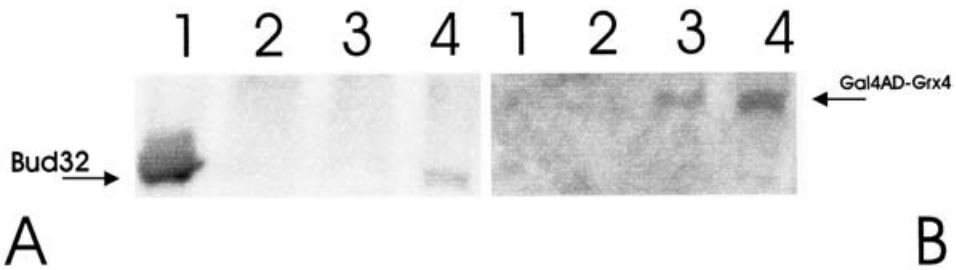

Figure 3 Analysis of the interaction of Bud32 with Grx4 by co-immunoprecipitation assay

Co-immunoprecipitation confirms the interaction between Bud32 and Grx4. Wild-type and Δ*BUD32* yeast cells were transformed with the pACTII and pACTII-Grx4 plasmids and grown on selective medium. Immunoprecipitation with anti-HA antibody of the Gal4 AD–HA–Grx4 fusion protein was followed by SDS/PAGE and Western blot. Lanes 1, recombinant Bud32–His_6; lanes 2, immunoprecipitate from wild-type yeast cells transformed with the pACTII vector without insert; lanes 3, immunoprecipitate from Δ*BUD32* cells transformed with the pACTII-Grx4 plasmid; lanes 4, immunoprecipitate from wild-type cells transformed with the pACTII-Grx4 vector. (**A**) Western blot with anti-Bud32 antibody; (**B**) Western blot with anti-HA antibody.

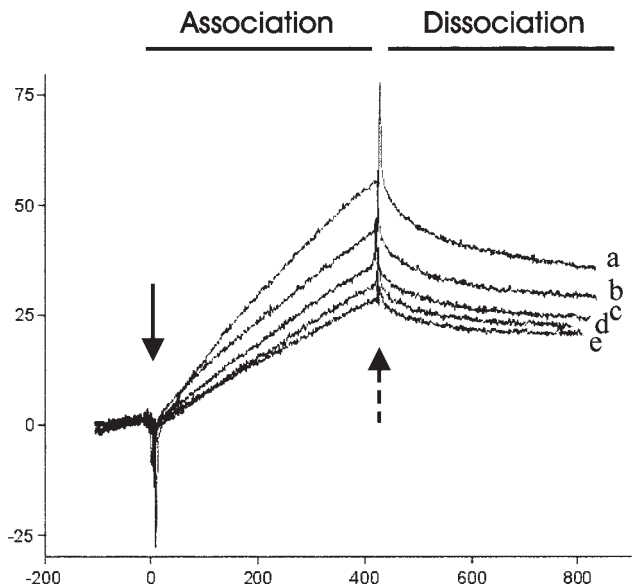

Figure 4 Physical interaction of Bud32 with Grx4 by SPR assay (BIAcore)

Superimposed sensorgrams, obtained by injection of different concentrations of Grx4 solutions (trace a, 2 μM; trace b, 1 μM; trace c, 0.8 μM; trace d, 0.6 μM; trace e, 0.4 μM) over a sensor surface where Bud32 was covalently immobilized, are shown. The SPR signal (expressed in resonance units) is already subtracted from the response obtained over a control surface, and normalized to a baseline of 0 resonance units. Injections started at time 0, as indicated by the solid arrow, and the ascending part of the curves corresponds to the association phase. The dashed arrow indicates the end of the injections, when protein solutions were replaced by the buffer and dissociation phase started. The kinetic parameters of the Bud32–Grx4 association evaluated by SPR analysis are $k_{ass} = 118.5\ M^{-1} \cdot s^{-1}$, $k_{diss} = 1.05 \times 10^{-3}\ s^{-1}$ and $K_d = 8.88 \times 10^{-6}$ M.

that immunoprecipitation of Grx4 by the anti-HA antibody (Figure 3B, lane 4) induces the co-precipitation of Bud32 (recognized by anti-Bud32 antibodies) as well (Figure 3A, lane 4).

The third technique was by SPR analysis: recombinant Bud32 purified from *E. coli* was covalently immobilized on the BIAcore Sensor Chip and a solution containing the Grx4 recombinant protein at different concentrations was allowed to flow over the chip. The resulting sensorgram shows that Bud32 and Grx4 are able to interact (Figure 4), so definitely providing the demonstration that the interaction between the two proteins is direct. The SPR analysis also allows estimation of the kinetic parameters of the interaction between the two proteins ($K_D = 8.88 \times 10^{-6}$ M). The interaction was confirmed in a reciprocal experiment in which the Grx4 protein was immobilized on the BIAcore Sensor Chip and a Bud32 solution was allowed to flow over it (results not shown).

Fourth, it should be mentioned that Grx4 was identified in protein complexes with Bud32 by an independent study based on high-throughput MS analysis [38].

Domains implicated in the interaction

Based on the two-hybrid results, the interaction between Bud32 and Grx4 appears to be mediated by the C-terminal region of Grx4, given the presence of this region in all the interacting clones identified by the two-hybrid approach and, in particular, the presence in one of the clones of only the last 100 amino acids (containing the catalytic CGFS site).

In order to map the region of Bud32 involved in the interaction, we prepared three different truncated forms of the Bud32 protein fused to the Gal4 BD domain (expressing amino acids 1–86, 1–196 and 87–STOP of Bud32) and used them in two-hybrid experiments to ascertain if they were still able to interact with the Grx4 protein (expressed by one of the Gal4 AD fusion plasmids). None of the truncated forms of Bud32 was able to interact, suggesting that either the overall 3D conformation is required or that the truncated peptides were unstable.

Bud32 phosphorylates Grx4 *in vitro*

We have tested the ability of the Bud32 protein kinase to phosphorylate Grx4, using the two recombinant proteins expressed and purified from *E. coli* in a phosphotransferase *in vitro* assay. As shown in Figure 5, Grx4 is phosphorylated in the presence of the wild-type form of Bud32 and not in the presence of a catalytically inactive Bud32 mutant, D161A [5], thus demonstrating the specificity of the phosphotransferase reaction. Phosphorylation linearly reaches 0.01–0.02 mol of phosphate/mol of Grx4 after 15 min of incubation, but it can be increased significantly by prolonging incubation and adding fresh aliquots of Bud32 at regular times: after three successive additions of Bud32 and 90 min incubation a stoichiometry of 0.15 mol of phosphate/mol of Grx4 has been observed. The kinetic parameters of the reaction have been calculated also (see Figure 6 and insets therein). While the submicromolar K_m value is consistent with the physiological relevance of Grx4 phosphorylation by Bud32, the V_{max} value corroborates the view that the catalytic efficiency of Bud32 is extremely low. Indeed the k_{cat} values of Bud32, determined with peptide substrates [4,5], are 4–5 orders of magnitude lower than those displayed in comparable assays by typical protein kinases, notably PKA and protein kinase CKII, probably owing to a

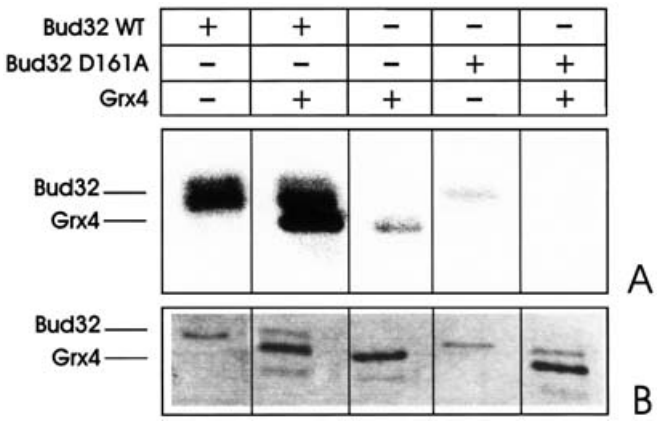

Figure 5 Grx4 phosphorylation by the Bud32 protein kinase

Approx. 1 μg of the recombinant Grx4 protein (1.8 μM final concentration) was subjected to phosphorylation by [γ-^{32}P]ATP in the presence of approx. 200 ng of the recombinant Bud32 proteins (0.3 μM final concentration), either wild-type or mutant D161A, as described in the Experimental section. (**A**) Autoradiography; (**B**) Coomassie Brilliant Blue staining.

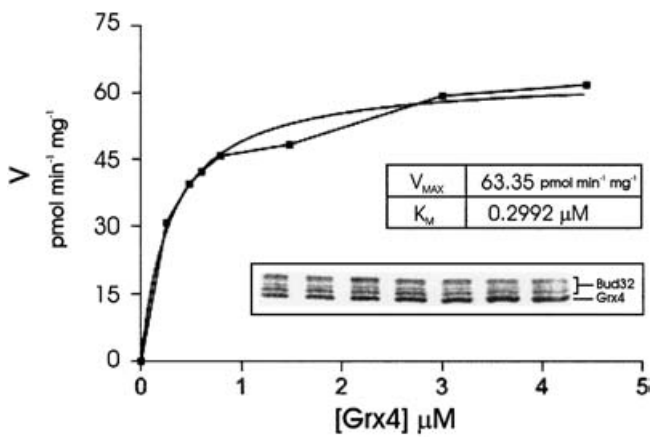

Figure 6 Determination of kinetic constants for Grx4 phosphorylation by Bud32

Phosphorylation was performed and estimated by incubation of Grx4 (0–5 μM) in the presence of Bud32 protein kinase (0.3 μM) at 37 °C for 15 min in 20 μl of a medium containing 50 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$ and 25 μM [γ-^{32}P]ATP (specific radioactivity, 2000 c.p.m./pmol).

number of abnormal features in the catalytic core of Bud32, as discussed elsewhere [5]. Especially remarkable in this respect is the poor conservation of the catalytic loop, where an essential lysyl residue conserved in all Ser/Thr protein kinases (Lys-168 in PKA), responsible for the transfer of the γ phosphate of ATP to the phosphoacceptor residue, is replaced by threonine (Thr-163) [5]. Mutation of PKA Lys-168 to alanine results in a 99.7% drop in catalytic efficiency [39], whereas mutation of Bud32 Thr-163 to alanine has no effect [5]. It can be concluded therefore that the alteration of the catalytic loop, with special reference to the lack of the essential lysine, is largely responsible for the low catalytic activity of Bud32. Whether such a low phosphotransferase activity reflects the fact that it is not crucially required for the physiological role of Bud32 remains an open question. The observation, reported above, that mutants which have almost completely lost catalytic activity are nevertheless able to partially complement the lack of Bud32 (see Figure 1) may argue in favour of this hypothesis. On the other hand the same experiments clearly show that complementation by these defective mutants is only partial, as compared with the wild type, consistent with a still important physiological role of Bud32 phosphotransferase activity.

In order to identify the residues phosphorylated by Bud32, phosphorylated Grx4 was digested with trypsin and the resulting peptides analysed by MALDI–time-of-flight MS. An aliquot of the tryptic digest was chemically phosphorylated by $Ba(OH)_2$. A comparison between the spectrum obtained with the tryptic peptides before and after chemical dephosphorylation (Figure 7, top panel) allowed us to identify one chemically dephosphorylated peptide corresponding to residues 124–148 (STSDEESSGSSDD-EEDETEEEINAR). The peptide contains six serines and two threonines, all possible targets of phosphorylation by Bud32. Further analysis by electrospray ionization-tandem MS of the chemically dephosphorylated peptides showed that the main target of Bud32 is Ser-134 (underlined above; Figure 7, bottom panel). A very weak phosphorylation of other seryl residue(s) in the same peptide has been also observed. Among these, Ser-133 has been identified as responsible, together with Ser-134, for the presence of a minimal amount of a bis-phosphorylated form of the 124–148 peptide. Note that the presence of several glutamic and aspartic acid residues surrounding Ser-134/Ser-133 corroborates the concept that Bud32 is an acidophilic Ser/Thr protein kinase [4]. Since the MALDI analysis showed a percentage of coverage of protein which is about 90%, we cannot exclude that the Bud32 protein kinase could phosphorylate different regions of Grx4 as well, but our data strongly indicate Ser-134 as the main phosphorylation site.

The location of the Ser-134 residue outside the C-terminal region of Grx4, responsible for association with Bud32 in the two-hybrid analysis (downstream from residue 148; see Table 1), supports the view that physical interaction with and phosphorylation by Bud32 are separate events, a conclusion reinforced by the significantly different values of the K_m for the phosphotransferase reaction (0.34 μM) and the K_d value calculated from the BIAcore analysis (8.8 μM). Note that a similar behaviour has been recently reported in the case of p53 phosphorylation by and interaction with Bud32 [11]. Associations between thiol-disulphide oxidoreductases and protein kinases have been observed in mammals [35,36] and a notable example is the association between human protein kinase Cθ and the putative oxidoreductase PICOT (protein kinase C-interacting cousin of thioredoxin), which is structurally similar to Grx4. However, in this case no phosphorylation of PICOT by protein kinase C could be observed [37]. Phosphorylation of proteins of the thiol-disulphide oxidoreductase family has been reported already, e.g. the phosphorylation of a 57 kDa rat protein (p57) related to protein disulphide isomerase by the Lyn tyrosine kinase is required for its association with the SH2 domain of the kinase [40].

Subcellular localization of the Bud32 and Grx4 proteins

In order to define the cellular localization of the two interacting proteins Bud32 and Grx4 both were expressed as green fluorescent fusion proteins. The fused *GFP–BUD32* sequence, expressing the Bud32 protein fused to the C-terminus of GFP, was placed on a yeast expression plasmid under the control of the methionine-repressible *MET25* promoter [20]. Wild-type and Δ*BUD32* yeast cells were transformed with GFP–Bud32 or GFP alone and observed at the microscope for GFP signals. In contrast to the cytoplasmic localization of the GFP alone, the majority of cells expressing GFP–Bud32 showed a strong fluorescence which

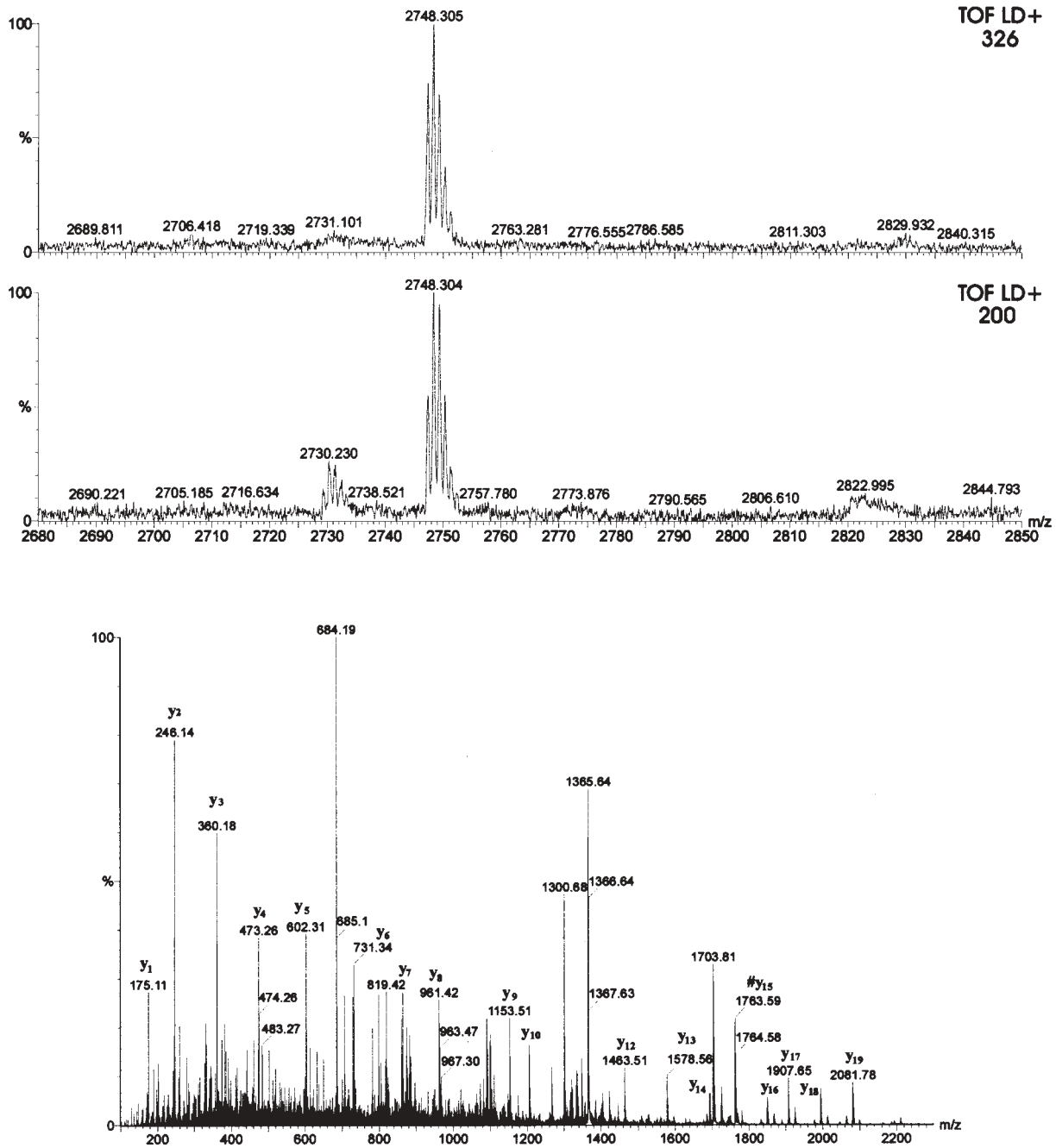

Figure 7 MS analysis of Grx4 phosphorylated by Bud32

Top panel: the upper spectrum shows a portion of the MALDI–time-of-flight (TOF) spectrum [laser desorption positive mode ionization (LD+)] of the tryptic peptides obtained after digestion of the phosphorylated Grx4 protein. The non-phosphorylated fraction of the 124–148 peptide (molecular mass 2747.3 Da) is evident, while the corresponding phosphopeptide (molecular mass 2827.1 Da) was not detected. The lower spectrum shows the situation after treatment of the tryptic digest with $Ba(OH)_2$. A new signal corresponding to chemically dephosphorylated 124–148 peptide is now evident (molecular mass 2729.3 Da). Bottom panel: product ion spectrum of the doubly charged, chemically dephosphorylated peptide Grx4 124–148. A complete *y*-series allowed the identification of Ser-134 as the main phosphorylation site. The signal at 1763.59 Da ($\#y_{15}$) clearly indicates that Ser-134 was subjected to dephosphorylation under basic conditions. The diagnostic peaks due to the possible dephosphorylation of other Thr or Ser residues within the same peptide are either not present or very weak.

co-localizes with the chromosomal DNA visualized by DAPI (4′,6-diamidino-2-phenylindole-dihydrochloride; Figure 8). This indicates that the fusion protein GFP–Bud32 is mainly located in the nuclear compartment of the cell. No differences in the fluorescence patterns were observed if the fusion protein was expressed at a low level (in the presence of methionine) or overexpressed, nor if the endogenous Bud32 protein was present or not (results not shown). Figure 8 shows that also the GFP–Grx4 fusion protein, similarly to GFP–Bud32, predominantly localizes to the nucleus, in agreement with the two-hybrid results that indicate the interaction between the two proteins. It is an interesting reminder that the human homologue of Bud32, PRPK, has also been shown to localize in the nucleus, and this has been correlated with the presence of a nuclear localization signal in the PRPK protein [12]. In Bud32, however, a similar sequence is not present, and a GFP fusion with a truncated Bud32 protein,

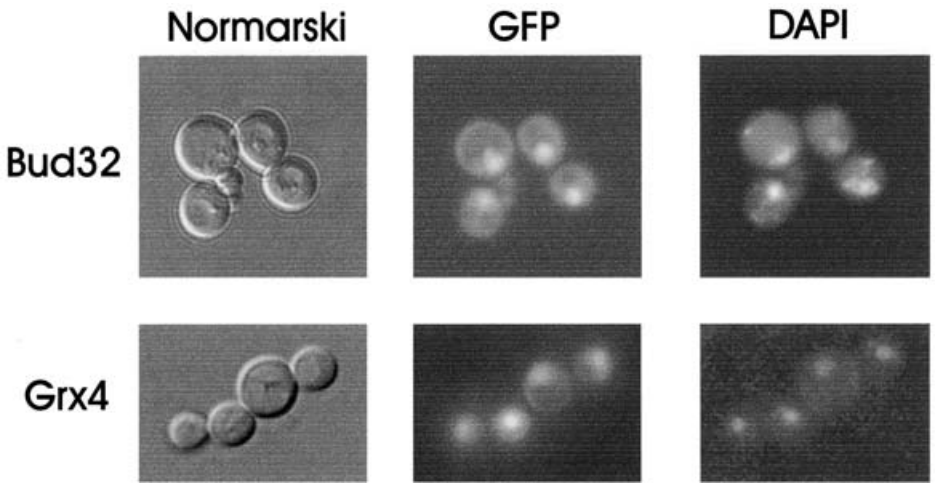

Figure 8 Nuclear localization of Bud32 and Grx4

Yeast cells of wild-type strain were transformed with the Bud32-pUG36 or Grx4-pUG36 plasmids and grown on selective medium. Samples harvested from liquid cultures of exponentially growing transformed cells have been observed by fluorescence microscopy to detect the GFP and DAPI (4′,6-diamidino-2-phenylindole-dihydrochloride) fluorescence signals.

lacking a C-terminal basic stretch, which could also represent a nuclear localization signal, still localizes to the nucleus (results not shown). Thus the structural basis for the nuclear localization of Bud32 remains unclear.

Conclusions

In this work we provide evidence that the phosphotransferase activity of the *S. cerevisiae* atypical protein kinase piD261/Bud32, previously demonstrated *in vitro* [3,5], is relevant to the function of the protein *in vivo*, as judged from the functional complementation assays using catalytically inactive mutants of piD261/Bud32. The result points out that, in addition to its protein kinase activity, piD261/Bud32 may have other cellular roles, probably correlated to its ability of interaction with other proteins. The relevance of Bud32 as an interacting protein in yeast was corroborated by the outcome of the two-hybrid approach, that has in fact identified several proteins able to associate with Bud32; in particular a Grx (Grx4), a putative glycoprotease (Ykr038/Kae1) and two proteins encoded by the *IMD* gene family seem most plausible interactors. All of them were also found, by an independent approach [38], among a group of about 30 proteins that co-precipitate with Bud32. In this work, we concentrated on Grx4. This protein has been shown by different methods to directly interact with piD261/Bud32 both *in vitro* and *in vivo*. We have also obtained evidence that Grx4 is phosphorylated by Bud32 *in vitro* with a K_m value denoting high affinity and we identified Ser-134 as the main phosphorylation site. This is present in a highly acidic stretch, in agreement with the previous characterization of Bud32 as an acidophilic protein kinase [4]. The location of the phosphorylation site upstream from the C-terminal minimal region sufficient for interaction with Bud32 (amino acids 148–STOP) supports the idea that physical interaction with and phosphorylation by Bud32 are distinct events, thus enforcing the relevance of the interaction properties for the Bud32 cellular function(s). The observed biochemical relationship between Bud32 and Grx4 strongly suggests that Grx4 might be one of the physiological targets of Bud32, and points to a possible role of this kinase in the control of the redox state maintenance, by acting as a regulator of Grx4 activity. The phosphorylation of Grx4 by Bud32 is also in agreement with the observed localization of both Bud32 and Grx4 in the nucleus, and points to a concerted role of the two proteins in this compartment, where the redox state is critical for the function of many transcription factors.

Finally, the interaction of Bud32 with Ykr038/Kae1, a putative protease essential to cell life, is of particular interest as in some archaebacteria species the sequence homologous to Bud32 and Ykr038 are fused in a polypeptide encoded by a single gene, supporting the view that functional interactions between members of the Bud32 and Ykr038 families play a crucial role which is highly conserved along the evolutionary scale.

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REFERENCES

1 Leonard, C., Aravind, L. and Koonin, E. V. (1998) Novel families of putative protein kinases in bacteria and archaea: evolution of the "eukaryotic" protein kinase superfamily. Genome Res. **8**, 1038–1047
2 Clemente, M. L., Sartori, G., Cardazzo, B. and Carignani, G. (1997) Analysis of an 11.6 kb region from the right arm of chromosome VII of *Saccharomyces cerevisiae* between the *RAD2* and the *MES1* genes reveals the presence of three new genes. Yeast **13**, 287–290
3 Stocchetto, S., Marin, O., Carignani, G. and Pinna, L. A. (1997) Biochemical evidence that *Saccharomyces cerevisiae YGR262c* gene, required for normal growth, encodes a novel Ser/Thr-specific protein kinase. FEBS Lett. **414**, 171–175
4 Facchin, S., Sarno, S., Marin, O., Lopreiato, R., Sartori, G. and Pinna, L. A. (2002) Acidophilic character of yeast piD261/Bud32, a putative ancestor of eukaryotic protein kinases. Biochem. Biophys. Res. Commun. **296**, 1366–1371
5 Facchin, S., Lopreiato, R., Stocchetto, S., Arrigoni, G., Cesaro, L., Marin, O., Carignani, G. and Pinna, L. A. (2002) Structure-function analysis of yeast piD261/Bud32, an atypical protein kinase essential for normal cell life. Biochem. J. **364**, 457–463
6 Sartori, G., Mazzotta, G., Stocchetto, S., Pavanello, A. and Carignani, G. (2000) Inactivation of six genes from chromosomes VII and XIV of *Saccharomyces cerevisiae* and basic phenotypic analysis of the mutant strains. Yeast **16**, 255–265
7 Briza, P., Bogengruber, E., Thur, A., Rutzler, M., Munsterkotter, M., Dawes, I. W. and Breitenbach, M. (2002) Systematic analysis of sporulation phenotypes in 624 non-lethal homozygous deletion strains of *Saccharomyces cerevisiae*. Yeast **19**, 403–422

8 Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O. and Herskowitz, I. (1998) The transcriptional program of sporulation in budding yeast. Science **282**, 699–705

9 de Groot, P. W. J., Ruiz, C., Vázquez de Aldana, C. R., Duenas, E., Cid, V. J., Del Rey, F. et al. (2001) A genomic approach for the identification and classification of genes involved in cell wall formation and its regulation in *Saccharomyces cerevisiae*. Comp. Funct. Genom. **2**, 124–142

10 Ni, L. and Snyder, M. (2001) A genomic study of the bipolar bud site selection pattern in *Saccharomyces cerevisiae*. Mol. Biol. Cell. **12**, 2147–2170

11 Facchin, S., Lopreiato, R., Ruzzene, M., Marin, O., Sartori, G., Götz, C., Montenarh, M., Carignani, G. and Pinna, L. A. (2003) Functional homology between yeast piD261/Bud32 and human PRPK: both phosphorylate p53 and PRPK partially complements piD261/Bud32 deficiency. FEBS Lett. **549**, 63–66

12 Abe, Y., Matsumoto, S., Wei, S., Nezu, K., Miyoshi, A., Kito, K., Ueda, N., Shigemoto, K., Hitsumoto, Y., Nikawa, J. and Enomoto, Y. (2001) Cloning and characterization of a p53-related protein kinase expressed in interleukin-2-activated cytotoxic T-cells, epithelial tumor cell lines, and the testes. J. Biol. Chem. **276**, 44003–44011

13 Bonneaud, N., Ozier-Kalogeropoulos, O., Li, G., Labouesse, M., Minvielle-Sebastia, L. and Lacroute F. (1991) A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae/E. coli* shuttle vectors. Yeast **7**, 609–615

14 Stotz, A. and Linder, P. (1990) The *ADE2* gene from *Saccharomyces cerevisiae*: sequence and new vectors. Gene **95**, 91–98

15 Gietz, R. D. and Woods, R. A. (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. **350**, 87–96

16 Fromont-Racine, M., Rain, J. C. and Legrain, P. (1997) Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. Nat. Genet. **16**, 277–282

17 Williams, B. A., Sillaots, S., Tsang, A. and Storms, R. (1996) Isolation by genetic complementation of two differentially expressed genes for beta-isopropylmalate dehydrogenase from *Aspergillus niger*. Curr. Genet. **30**, 305–311

18 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

19 Ruzzene, M., Brunati, A. M., Sarno, S., Donella-Deana, A. and Pinna, L. A. (1999) Hematopoietic lineage cell specific protein 1 associates with and down-regulates protein kinase CK2. FEBS Lett. **461**, 32–36

20 Niedenthal, R. K., Riles, L., Johnston, M. and Hegemann, J. H. (1996) Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast **12**, 773–786

21 Holmes, C. F. (1987) A new method for the selective isolation of phosphoserine-containing peptides. FEBS Lett. **215**, 21–24

22 Meyer, H. E., Swiderek, K., Hoffmann-Posorske, E., Korte, H. and Heilmeyer, Jr, L. M. (1987) Quantitative determination of phosphoserine by high-performance liquid chromatography as the phenylthiocarbamyl-S-ethylcysteine. Application to picomolar amounts of peptides and proteins. J. Chromatogr. **397**, 113–121

23 Resing, K. A., Johnson, R. S. and Walsh, K. A. (1995) Mass spectrometric analysis of 21 phosphorylation sites in the internal repeat of rat profilaggrin, precursor of an intermediate filament associated protein. Biochemistry **34**, 9477–9487

24 Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai, B. S. and Mische, S. M. (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis **20**, 601–605

25 Hunt, D. F., Yates, 3rd, J. R., Shabanowitz, J., Winston, S. and Hauer, C. R. (1986) Protein sequencing by tandem mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. **83**, 6233–6237

26 Kukuruzinska, M. A. and Lennon, K. (1995) Diminished activity of the first N-glycosylation enzyme, dolichol-P-dependent N-acetylglucosamine-1-P transferase (GPT), gives rise to mutant phenotypes in yeast. Biochim. Biophys. Acta **1247**, 51–59

27 Wysocki. R., Van Dyck, E., Fairhead, C. and Foury, F. (1999) Mass-murdering: deletion of twenty-three ORFs from *Saccharomyces cerevisiae* chromosome XI reveals five genes essential for growth and three genes conferring detectable mutant phenotype. Gene **229**, 37–45

28 Deutschbauer, A. M., Williams, R. M., Chu, A. M. and Davis, R. W. (2002) Parallel phenotypic analysis of sporulation and postgermination growth in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **99**, 15530–15535

29 Zimmermann, A. G., Gu, J. J., Laliberte, J. and Mitchell, B. S. (1998) Inosine-5′-monophosphate dehydrogenase: regulation of expression and role in cellular proliferation and T lymphocyte activation. Prog. Nucleic. Acid. Res. Mol. Biol. **61**, 181–209

30 Shaw, R., Wilson, J., Smith, K. and Reines, D. (2001) Regulation of an IMP dehydrogenase gene and its overexpression in drug-sensitive transcription elongation mutants of yeast. J. Biol. Chem. **276**, 32905–32916

31 Abdullah, K. M., Lo, R. Y. C. and Mellors, A. (1991) Cloning, nucleotide sequence, and expression of the *Pasteurella haemolytica* A1 glycoprotease gene. J. Bacteriol. **173**, 5997–5603

32 Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H. et al. (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science **285**, 901–906

33 Rodrìguez-Manzaneque, M. T., Ros, J., Cabiscol, E., Sorribas, A. and Herrero, E. (1999) Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19**, 8180–8190

34 Rodrìguez-Manzaneque, M. T., Tamarit, J., Belli, G., Ros, J. and Herrero, E. (2002) Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. Mol. Biol. Cell. **13**, 1109–1121

35 Morel, Y. and Barouki, R. (1999) Repression of gene expression by oxidative stress. Biochem. J. **342**, 481–496

36 Finkel, T. and Holbrook, N. J. (2000) Oxidants, oxidative stress and the biology of ageing. Nature (London) **408**, 239–247

37 Witte, S., Villalba, M., Bi, K., Liu, Y., Isakov, N. and Altman, A. (2000) Inhibition of the c-Jun N-terminal kinase/AP-1 and NF-kB pathways by PICOT, a novel protein kinase C-interacting protein with a thioredoxin homology domain. J. Biol. Chem. **275**, 1902–1909

38 Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutilier, K. et al. (2002) Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature (London) **415**, 180–183

39 Gibbs, C. S. and Zoller, M. J. (1991) Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. J. Biol. Chem. **266**, 8923–8931

40 Donella-Deana, A., James, P., Staudenmann, W., Cesaro, L., Marin, O., Brunati, A. M., Ruzzene, M. and Pinna, L. A. (1996) Isolation from spleen of a 57-kDa protein substrate of the tyrosine kinase Lyn. Identification as a protein related to protein disulfide-isomerase and localisation of the phosphorylation sites. Eur. J. Biochem. **235**, 18–25

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