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The SLT2(MPK1) MAP kinase is activated during periods of polarized cell growth in yeast

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The SLT2(MPK1) mitogen-activated protein kinase signal transduction pathway has been implicated in several biological processes in *Saccharomyces cerevisiae*, including the regulation of cytoskeletal and cell wall structure, polarized cell growth, and response to nutrient availability, hypo-osmotic shock and heat shock. We examined the conditions under which the SLT2 pathway is activated. We found that the SLT2 kinase is tyrosine phosphorylated and activated during periods in which yeast cells are undergoing polarized cell growth, namely during bud formation of vegetative cell division and during projection formation upon treatment with mating pheromone. BCK1(SLK1), a MEK kinase, is required for SLT2 activation in both of these situations. Upstream of BCK1(SLK1), we found that the STE20 kinase was required for SLT2 activation by mating pheromone, but was unnecessary for its activation during the vegetative cell cycle. Finally, SLT2 activation during vegetative growth was partially dependent on CDC28 in that the stimulation of SLT2 tyrosine phosphorylation was significantly reduced directly after a temperature shift in *cdc28* ts mutants. Our data are consistent with a role for SLT2 in promoting polarized cell growth.

Keywords: cell cycle/mating pheromone signal transduction/ SLT2(MPK1) MAP kinase activation/yeast polarized growth

Introduction

MAP (mitogen-activated protein) kinases are involved in diverse signal transduction pathways (Nishida and Gotoh, 1993; Cano and Mahadevan, 1995; Herskowitz, 1995). The MAP kinases are activated by phosphorylation on nearby tyrosine and threonine residues by dual-specificity MAPK kinases (also known as MEKs) which are in turn phosphorylated and activated by MAPKK (MEK) kinases. In the yeast *Saccharomyces cerevisiae*, at least four kinase cascade modules of this type exist (Herskowitz, 1995; Levin and Errede, 1995). SLT2(MPK1) is a MAP kinase isoform that is thought to be activated by the kinases MKK1 and MKK2, which in turn may be activated by BCK1(SLK1) (Irie *et al.*, 1993; Kamada *et al.*, 1995). The SLT2(MPK1) kinase pathway has been implicated in maintaining the integrity of the cytoskeleton and cell wall, in polarized cell growth, and in responses to nutrient availability, hypo-osmotic shock and heat shock (Torres *et al.*, 1991; Costigan *et al.*, 1992, 1994; Irie *et al.*, 1993; Lee *et al.*, 1993; Mazzoni *et al.*, 1993; Costigan and Snyder, 1994; Kamada *et al.*, 1995). How SLT2 influences these various physiological processes is not yet known.

We isolated an *slt2* mutant as an enhancer of the division defects associated with *cdc28* alleles that are partially defective in carrying out the G_1–Start transition (Mazzoni *et al.*, 1993). Activation of the CDC28 kinase at Start is required for bud formation, DNA replication and spindle pole body duplication (Pringle and Hartwell, 1981). Our analysis of *slt2* mutants led us to propose that SLT2 may function downstream or in parallel with CDC28 in stimulating polarized growth. In this work, we tested our hypothesis by exploring the conditions in which the SLT2 kinase is activated. We find that SLT2 is indeed activated during periods in which yeast cells undergo polarized cell growth.

Results

We introduced a haemagglutinin (HA) epitope into SLT2 (Figure 1A) so that we could specifically immunoprecipitate the tagged protein. The SLT2HA protein is functional as it can complement the temperature-sensitive growth defect of an *slt2::HIS3* mutant when expressed from a centromeric plasmid vector (data not shown). We wanted to test the hypothesis that the SLT2 pathway is activated specifically during the cell cycle during periods of polarized cell growth (Mazzoni *et al.*, 1993). SLT2 kinase activation was followed by determining the amount of phosphotyrosine associated with the protein. All MAP kinases examined to date are activated by their phosphorylation at a closely spaced tyrosine and threonine residue by dual-specificity MAPK kinases (Nishida and Gotoh, 1993; Cano and Mahadevan, 1995). Mutagenesis studies have shown that the corresponding residues of SLT2, Thr190 and Tyr192, are important for its function (Lee *et al.*, 1993; Martin *et al.*, 1993). In the course of these studies, we found that the SLT2 kinase is phosphorylated on tyrosine when yeast cells are treated with the α-mating pheromone and when cells are transferred to higher growth temperatures (Figure 1B and C). No phosphotyrosine was found associated with an SLT2HA-Y192F mutant in which Tyr192 had been changed to phenylalanine (Figure 1D).

Increased levels of SLT2 phosphotyrosine were correlated with a stimulation of the kinase activity associated with SLT2HA immunoprecipitates, as seen by their ability to phosphorylate myelin basic protein (MBP), a standard substrate of MAP kinases (Figure 2B). Phosphorylation

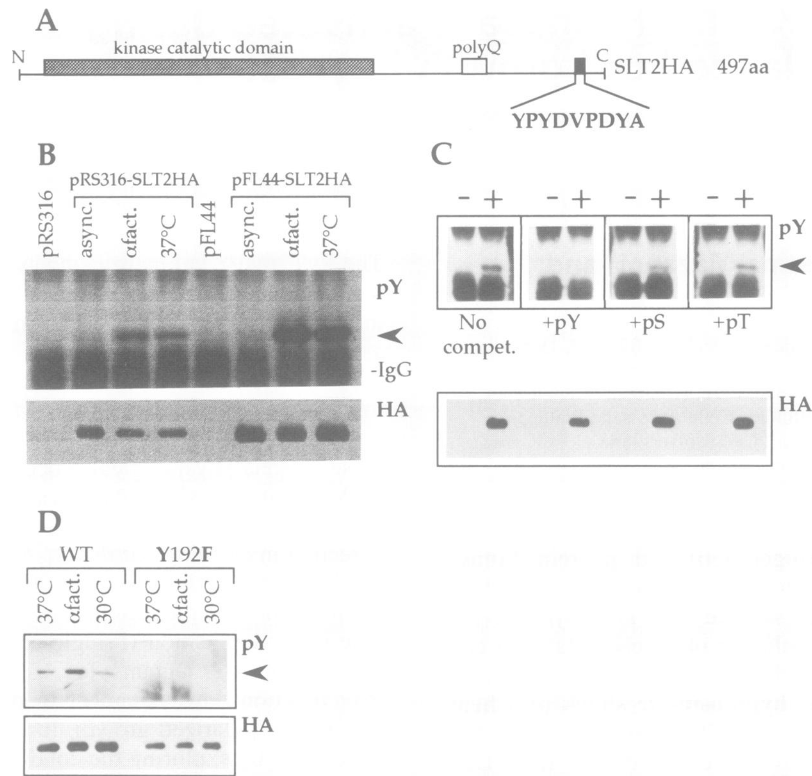

Fig. 1. HA epitope tagging of SLT2 and determination of its phosphotyrosine content. (**A**) Schematic diagram of important features of the SLT2HA protein sequence with the position of the inserted HA epitope tag (YPYDVPDYA). (**B**) Phosphotyrosine content of SLT2HA is stimulated by growth of cells at elevated temperatures or by treatment of cells with α-factor. SLT2HA was immunoprecipitated with monoclonal 12CA5 anti-HA antibodies from whole cell protein extracts prepared from wild-type strains (PZY142, PZY181) containing centromeric (pRS316-SLT2HA) or multicopy (pFL44-SLT2HA) plasmids. Cells were grown at 30°C (async.), treated for 3 h with 1 μM α-factor at 30°C (αfact.), or transferred from 30 to 37°C for 3 h (37°C). Control immunoprecipitations were also carried out with extracts from wild-type cells (PZY144, PZY143) at 30°C containing only the pRS316 or pFL44 vectors. Immunoprecipitated proteins were separated by SDS–PAGE and transferred to membranes. Membranes containing immunoprecipitated proteins were probed separately with anti-HA antibodies (HA) to quantify the amount of SLT2HA immunoprecipitated, and monoclonal 4G10 anti-phosphotyrosine antibodies (pY, arrowhead shows the position of phosphotyrosine-containing SLT2HA) to determine the amount of phosphotyrosine associated with SLT2HA. IgG indicates the position of the anti-HA immunoglobulin heavy chain that is recognized by the HRP-conjugated anti-mouse antibodies used to develop the Western blot (see Materials and methods).
(**C**) Demonstration that the monoclonal anti-phosphotyrosine antibodies specifically recognize phosphotyrosine. Protein extracts were prepared from wild-type cells (PZY142, PZY144) containing the plasmid pRS316-SLT2HA (+) or the vector pRS316 alone (–) after treating cells with 1 μM α-factor for 2 h at 30°C. Proteins were immunoprecipitated with 12CA5 anti-HA antibodies, electrophoresed on SDS–polyacrylamide gels and transferred to membranes. The membranes were then probed with monoclonal 4G10 anti-phosphotyrosine antibodies alone (No compet.), or in the presence of 2 mM phosphotyrosine (+pY), 2 mM phosphoserine (+pS) or 2 mM phosphothreonine (+pT). (**D**) The Y192F mutation of SLT2 eliminates tyrosine phosphorylation. Protein extracts were prepared from wild-type cells (CMY826) containing the plasmid pFL44-SLT2HA (WT) or pFL44-SLT2Y192F-HA (Y192F) during log phase growth at 30°C, after addition of 1 μM α-factor and incubation at 30°C for 3 h (αfact.), or after transfer from 30 to 37°C for 3 h. Immunoprecipitation of anti-HA tagged protein and Western blotting were performed as for (B).

of MBP was increased 3- to 4-fold when SLT2HA was immunoprecipitated from cells that were grown at 37°C for 2.5 h compared with SLT2HA immunoprecipitated from cells grown at 30°C. Phosphorylation of these same proteins was increased 6- to 8-fold when SLT2HA was immunoprecipitated from cells treated with α-factor for 2.5 h at 30°C compared with SLT2HA immunoprecipitated from untreated cells at 30°C. Mutation of SLT2 Lys54 to arginine inactivates the *in vivo* function of the kinase (Martin *et al.*, 1993) and we found that $SLT2^{K54R}HA$ immune complexes had no MBP kinase activity above that found in imunoprecipitates from a control strain that did not express HA-tagged SLT2 (Figure 2D).

A 57 kDa protein and a doublet of proteins migrating at ~62 kDa were also phosphorylated in SLT2HA immune complexes (Figure 2A). The 57 kDa protein is likely to be an immunoglobulin heavy chain, as this band is not observed when the immunoprecipitation is carried out with antibodies that have been covalently coupled to protein A–Sepharose beads (data not shown). The two bands at ~62 kDa are likely to be two phosphorylated forms of the SLT2HA kinase itself, since the doublet co-migrates with SLT2HA and both of these proteins are re-precipitated efficiently by anti-HA antibodies after phosphorylation *in vitro* and denaturation of protein complexes (data not shown; see Materials and methods). Although $SLT2^{K54R}HA$ immune complexes were completely defective for phosphorylation of MBP and the 57 kDa protein, a weak level of phosphorylation of the lower 62 kDa band was observed (Figure 2C). This band may be due to phosphorylation of $SLT2^{K54R}HA$ by a co-precipitating kinase. However, since this phosphorylation is weak compared with SLT2HA immunoprecipitations, we suggest that autophosphorylation contributes significantly to SLT2HA phosphorylation in these immune complex kinase reactions.

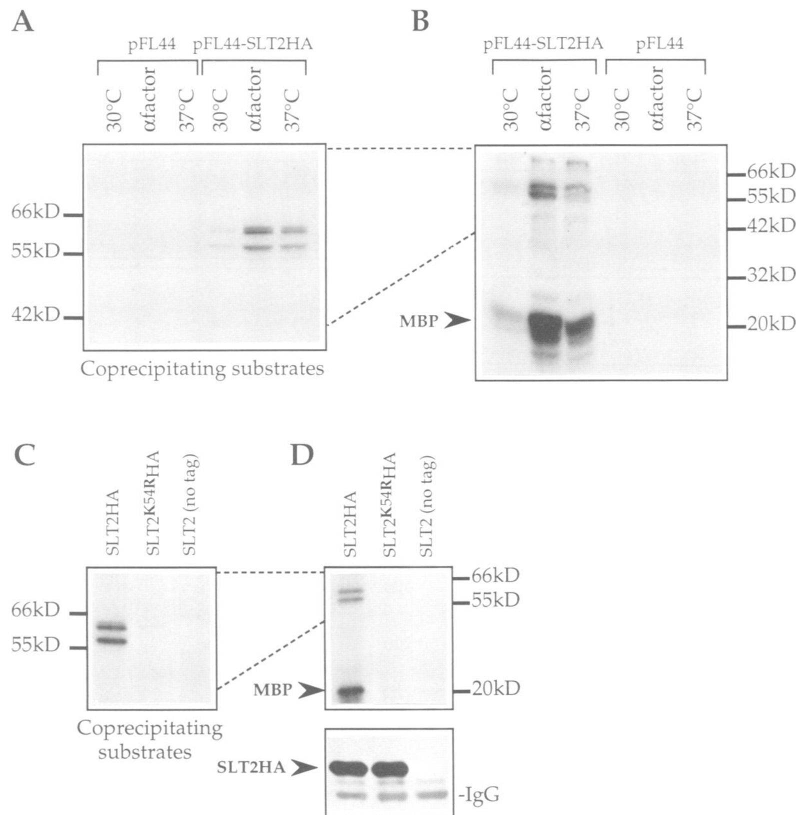

Fig. 2. Protein kinase activity associated with SLT2HA immunoprecipitates is increased after treatment of cells with α-factor or growth at elevated temperatures. (**A** and **B**) Proteins were immunoprecipitated with 12CA5 anti-HA antibodies from wild-type cells (PZY143, PZY181) containing the plasmids pFL44 or pFL44-SLT2HA. Cells were grown at 30°C, treated with α-factor at 30°C for 2.5 h (αfactor), or transferred from 30 to 37°C for 2.5 h (37°C). One-third of the immunoprecipitates was then incubated in the presence of [γ-^{32}P]ATP at 30°C for 15 min in order to phosphorylate co-precipitating substrates (A), another third was incubated with [γ-^{32}P]ATP + MBP at 30°C for 15 min in order to assay MBP kinase activity (B), and the final third was used for a Western blot to verify that the same quantity of SLT2HA was immunoprecipitated from the cells containing pFL44-SLT2HA (data not shown). The co-precipitating substrate reaction (A) was electrophoresed on a 10% polyacrylamide gel, whereas the MBP kinase reaction (B) was electrophoresed on a 15% polyacrylamide gel. (**C** and **D**) The K54R mutation eliminates the *in vitro* kinase activity of SLT2HA. Wild-type (CMY826) cells containing pFL44-SLT2HA, pFL44-SLT2K54RHA or pFL44-SLT2 (no tag), were treated with 1 μM α-factor for 3 h before preparing extracts for anti-HA immunoprecipitation. Immune complex reactions to assay phosphorylation of co-precipitating substrates (C) or MBP (D) were done as for (A) and (B). The lower panel of (D) shows a Western blot of the immunoprecipitations used to evaluate the quantity of wild-type or K54R mutant SLT2HA present in the different immune complexes.

SLT2 is phosphorylated on tyrosine during periods of polarized cell growth in the yeast cell cycle

We used synchronized yeast cell populations to determine whether SLT2 is activated at specific points of the cell division cycle. Cells were blocked in the G_1 phase with α-factor and then allowed to re-enter the cell cycle by removal of the pheromone. Synchronization was carried out at both 30 and 35°C, since we had previously noticed that overall levels of SLT2 tyrosine phosphorylation are increased at elevated temperatures (Figure 1B). As noted above, treatment with α-factor induces SLT2 tyrosine phosphorylation (Figure 3). This phosphorylation was lost within 8 min after removal of the pheromone. At both 30 and 35°C, SLT2 tyrosine phosphorylation reappeared just before or at the time that cells first began budding. SLT2 tyrosine phosphorylation was inhibited at mitosis of the first division following α-factor release and reappeared once again just before or at the time that cells began budding in the second cell cycle after α-factor removal. The major difference between 30 and 35°C concerns the cell cycle period in which SLT2 was tyrosine phosphorylated. At 30°C, SLT2 tyrosine phosphorylation was limited to a short period around the time that cells first began to bud. Bud formation at this time requires a highly polarized growth of the cell (Lew and Reed, 1993, 1995). At 35°C, SLT2 is phosphorylated on tyrosine over a large part of the cell cycle, but this phosphorylation is transiently repressed around the time of mitosis. Repression of SLT2 tyrosine phosphorylation is thus correlated with the depolarization of cell growth that occurs during mitosis (Lew and Reed, 1993, 1995). Further support for this conclusion comes from experiments in which we observed that SLT2 tyrosine phosphorylation is inhibited in cells treated with nocodazole, a drug which prevents yeast cells from completing mitosis by inhibiting tubulin polymerization (Jacobs *et al.*, 1988). Asynchronously growing cells at 30°C were treated with nocodazole for 1 h 30 min and 3 h 10 min. SLT2 tyrosine phosphorylation was seen to decrease as the fraction of cells blocked in mitosis increased (Figure 4). After removal of nocodazole, cells completed division and SLT2 tyrosine phosphorylation was greatly stimulated at the time that cells began rebudding.

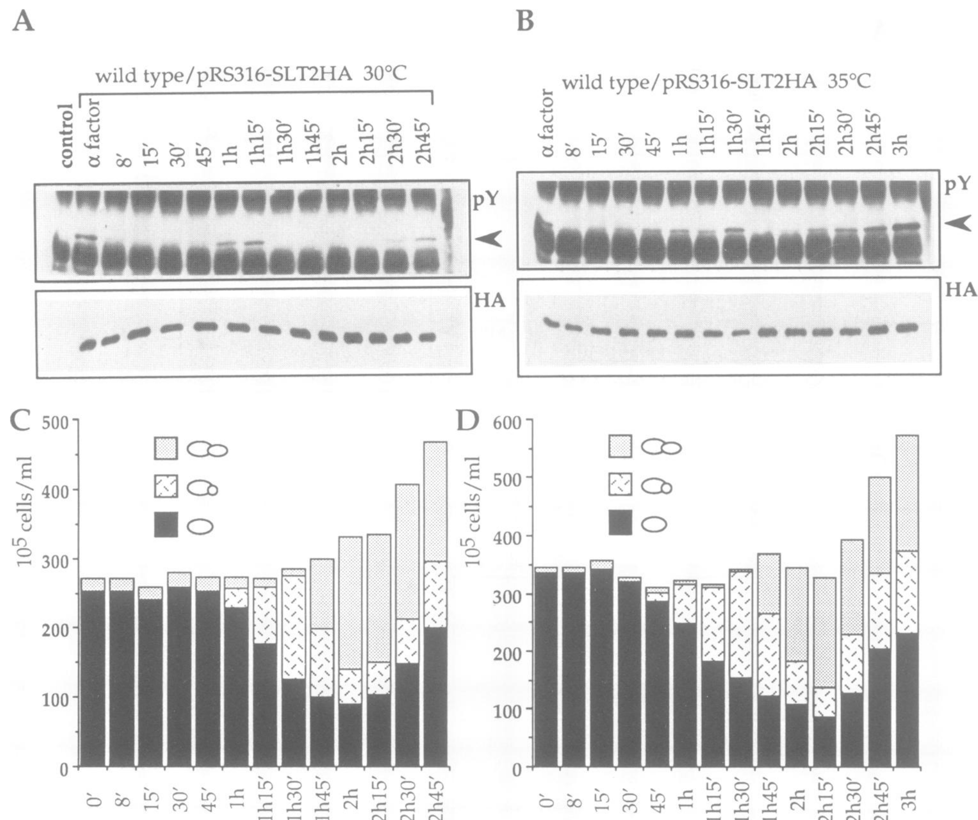

Fig. 3. SLT2 tyrosine phosphorylation is induced during periods of the cell division cycle in which cells are undergoing polarized growth and is repressed in mitosis. PZY142 (pRS316-SLT2HA) cells growing exponentially at 30°C (**A** and **C**) or 35°C (**B** and **D**) were treated with 1 μM α-factor until >90% of cells were without buds in G_1 phase. α-Factor was then removed and cells entered a synchronous division cycle. At the indicated time points, SLT2HA was immunoprecipitated (HA) and SLT2HA tyrosine phosphorylation (pY, arrowhead) was assayed (A and B) as described in Materials and methods, and the numbers of unbudded, small budded and large budded cells were counted (C and D). The control lane in (A) is an immunoprecipitation from a wild-type strain (PZY144) growing at 30°C that contained only the pRS316 vector.

Tyrosine phosphorylation of SLT2 requires the BCK1(SLK1) kinase

Genetic data have suggested that SLT2 activation requires the functionally redundant MKK1 and MKK2 kinases, which in turn would be activated by the BCK1(SLK1) kinase (Irie *et al.*, 1993; Kamada *et al.*, 1995). In wild-type cells, SLT2 tyrosine phosphorylation is stimulated by treating cells with α-factor at 24°C, or after shifting cells at 24 to 30 or 37°C (Figure 5, WT). In contrast, no tyrosine phosphorylation could be found associated with SLT2 in a *bck1(slk1)* deletion strain at 24°C, after treatment with α-factor at 24°C for 3 h, or after transfer of cells from 24 to 30 or 37°C for 3 h (Figure 5, SLK1Δ). The lack of SLT2 tyrosine phosphorylation in the *bck1(slk1)*/pRS316-SLT2HA strain is not due to loss of cell viability under these induction conditions: >90% of the cells were viable after a 3 h incubation with α-factor at 24°C, 85% were viable after a 3 h incubation at 30°C, and 64% were viable after a 3 h incubation at 37°C. Thus, activation of SLT2 by heat or α-factor treatment requires the BCK1(SLK1) kinase.

α-Factor-induced tyrosine phosphorylation requires the STE20 kinase

Yeast mating pheromones produce a G_1 phase arrest of cell division and changes in gene transcription through the activation of the FUS3/KSS1 MAP kinases (Schultz *et al.*, 1995). Current evidence indicates that FUS3/KSS1 are activated by the STE7 kinase which in turn is activated by the STE11 kinase (Errede *et al.*, 1993; Neiman and Herskowitz, 1994). Finally, the STE20 kinase is thought to activate this entire kinase cascade by an as yet undefined mechanism (Leberer *et al.*, 1992). The observation that α-factor cannot induce tyrosine phosphorylation of SLT2 in a *bck1* deletion mutant (Figure 5) suggests that STE11, the corresponding kinase in the FUS3/KSS1 cascade, cannot normally take the place of BCK1 in activating the MKK1/MKK2 kinases. Since both MAP kinase cascades are activated by α-factor, we examined the effect of a *ste20* disruption on SLT2 phosphorylation. Interestingly, α-factor treatment did not appreciably induce SLT2 tyrosine phosphorylation in a *ste20* mutant (Figure 6A). In the same mutant, a temperature shift induced a normal SLT2 phosphorylation (Figure 6A). Thus, STE20 represents an apparent branch point at which both the SLT2 and FUS3/KSS1 MAP kinase cascades are activated by α-factor.

Inactivation of the FUS3/KSS1 MAP kinase pathway decreases the transcription of a series of genes coding for components of this pathway (Schultz *et al.*, 1995). Expression of these genes is controlled by the STE12 transcription factor. Tyrosine phosphorylation of SLT2 was only slightly inhibited in a *ste12* disruption mutant (Figure 6B). Thus, induction of SLT2 tyrosine phos-

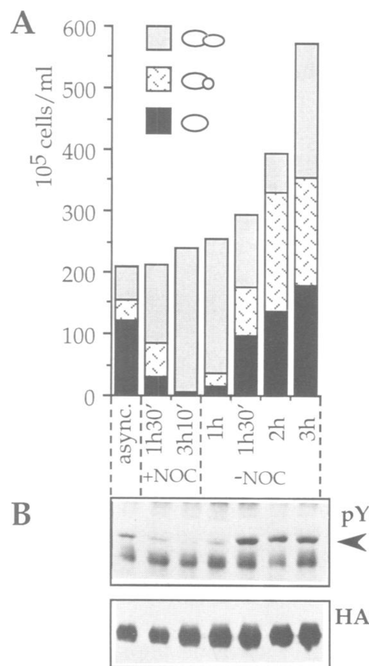

Fig. 4. Blocking cells in mitosis with nocodazole inhibits SLT2 tyrosine phosphorylation. Wild-type (SC55) diploid cells containing pFL44-SLT2HA growing exponentially in YPD at 30°C were treated with 15 µg/ml nocodazole by diluting from a stock solution at 11 mg/ml in DMSO. Cells were then incubated for 3 h 10 min, at which point >90% of the cells were blocked with a large bud in mitosis. The cells were then washed and resuspended in YPD to remove the nocodazole. At the indicated time points, an aliquot of cells was fixed with formaldehyde in order to determine the number of unbudded, small budded and large budded cells (**A**) and SLT2HA was immunoprecipitated (HA) and SLT2HA phosphotyrosine (pY, arrowhead) was determined (**B**) as described in Materials and methods.

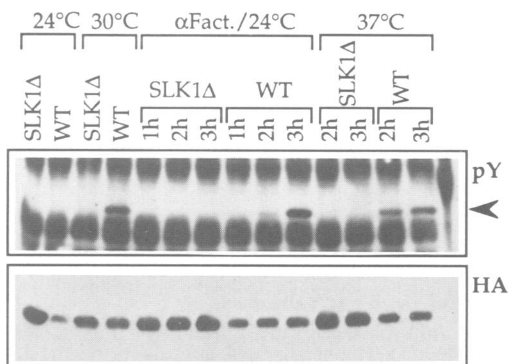

Fig. 5. SLT2 tyrosine phosphorylation is abolished in *bck1(slk1)* mutants. SLT2HA was immunoprecipitated (HA) and SLT2HA tyrosine phosphorylation (pY, arrowhead) was assayed (see Materials and methods) for wild-type/pRS316-SLT2HA (PZY142) cells and *slk1*/pRS316-SLT2HA (PZY184) cells growing exponentially at 24°C, after transfer from 24 to 30°C for 3 h (30°C), after transfer from 24 to 37°C for 2 or 3 h (37°C), or after treatment of cells growing at 24°C with α-factor for 1, 2 or 3 h (αFact./24°C).

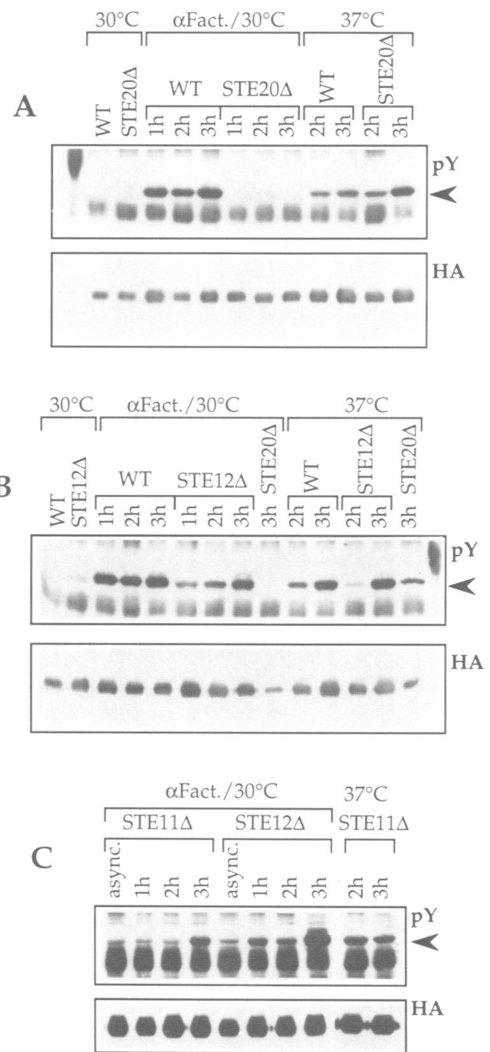

Fig. 6. α-Factor-induced SLT2 tyrosine phosphorylation is blocked in *ste20* mutants, but not in *ste12* or *ste11* mutants. (**A**) SLT2HA was immunoprecipitated (HA) and SLT2HA tyrosine phosphorylation (pY) was assayed (see Materials and methods) for wild-type/pRS424-SLT2HA (CMY912) and *ste20*/pRS424-SLT2HA (CMY913) cells growing exponentially at 30°C, after treatment with α-factor at 30°C for 1, 2 or 3 h (αFact./30°C), or after transfer from 30 to 37°C for 2 or 3 h (37°C). (**B**) SLT2HA was immunoprecipitated (HA) and SLT2HA tyrosine phosphorylation (pY) was assayed (see Materials and methods) for wild-type/pRS424-SLT2HA (CMY912) and *ste12*/pRS424-SLT2HA (CMY914) cells growing exponentially at 30°C, after treatment with α-factor at 30°C for 1, 2 or 3 h (αFact./30°C), or after transfer from 30 to 37°C for 2 or 3 h (37°C). For purposes of direct comparison, levels of SLT2 tyrosine phosphorylation are also shown in this panel for the *ste20*/pRS424-SLT2HA (CMY913) cells after treatment with α-factor for 3 h at 30°C and after transfer from 30 to 37°C for 3 h. (**C**) SLT2HA was immunoprecipitated (HA) and SLT2HA tyrosine phosphorylation (pY) was assayed (see Materials and methods) for *ste11*/pRS424-SLT2HA (CMY918) and *ste12*/pRS424-SLT2HA (CMY914) cells growing exponentially at 30°C, after treatment with α-factor at 30°C for 1, 2 or 3 h (αFact./30°C), or after transfer from 30 to 37°C for 2 or 3 h (37°C).

phorylation by α-factor does not require the transcriptional activation of genes controlled by STE12. We obtained further evidence that SLT2 tyrosine phosphorylation does not require the FUS3/KSS1 MAP kinase pathway by examining a *ste11* mutant. Deletion of *STE11* appreciably delays α-factor stimulation of SLT2 tyrosine phosphorylation, but a strong stimulation is nevertheless observed after 3 h of incubation (Figure 6C). Activation of SLT2 tyrosine phosphorylation after shifting cells from 30 to 37°C occurs normally in the *ste11* deletion mutant (Figure 6C).

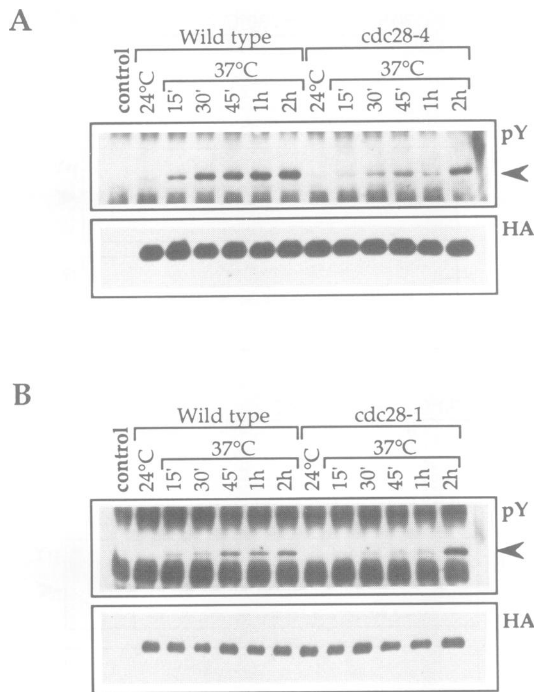

Fig. 7. SLT2 tyrosine phosphorylation is delayed in *cdc28* mutants. (**A**) SLT2HA was immunoprecipitated (HA) and SLT2HA tyrosine phosphorylation (pY) was assayed (see Materials and methods) for wild-type/pRS316-SLT2HA (PZY142) and *cdc28-4*/pRS316-SLT2HA (PZY171) cells growing exponentially at 24°C or after transfer from 24°C to 37°C for 15, 30, 45 min or 1 or 2 h (37°C). (**B**) As for (A), except that a *cdc28-1*/pRS316-SLT2HA (PZY173) strain was examined.

Tyrosine phosphorylation of SLT2 is delayed in cdc28 mutants

Inactivation of SLT2 enhances the phenotypic defects of *cdc28* mutants that are affected in their ability to trigger the Start transition (Mazzoni *et al.*, 1993). We were thus interested in determining whether activation of the SLT2 pathway is dependent on CDC28 function. The *cdc28-1* and *cdc28-4* mutants arrest cell division in the G_1 phase when transferred to the restrictive temperature of 37°C (Hartwell, 1973; Reed, 1980). An increase in SLT2 tyrosine phosphorylation could be detected within 15 min when wild-type cells were transferred from 24 to 37°C (Figure 7). This level of phosphorylation was maximal after 30–45 min of incubation at 37°C and remained elevated at this temperature. In contrast, when *cdc28-1* or *cdc28-4* mutants were transferred from 24 to 37°C, an equivalent increase in SLT2 tyrosine phosphorylation was only observed after 2 h of incubation (Figure 7). These results suggest that SLT2 tyrosine phosphorylation can occur through both CDC28-dependent and CDC28-independent pathways.

Discussion

SLT2 is activated during periods of polarized cell growth

Our previous phenotypic analysis of *slt2* mutants suggested that SLT2 is involved in stimulating polarized cell growth (Mazzoni *et al.*, 1993). Consistent with this interpretation, we find that SLT2 tyrosine phosphorylation and kinase activity are increased when yeast cells are undergoing polarized cell growth. In the yeast cell cycle, polarized cell growth for bud formation is triggered at the Start transition in G_1 phase upon activation of the CDC28 kinase by the CLN1 and CLN2 G_1 cyclins. The resulting bud growth remains largely apical until the CLB2 cyclin is made in G_2/mitosis (Lew and Reed, 1993, 1995). Our analysis of cells synchronized with α-factor showed that tyrosine phosphorylation was absent from SLT2 when cells were in mitosis. At 30°C, SLT2 was phosphorylated during a relatively brief period of the cell cycle at the time that cells first began to bud. In contrast, at 35°C, SLT2 tyrosine phosphorylation was maintained over a large fraction of the cell cycle with only a transient inhibition around the time of mitosis. Stimulation of SLT2 activity at elevated temperatures may reflect a greater need for this pathway in cytoskeletal integrity and polarized cell growth under these conditions (Costigan *et al.*, 1992; Mazzoni *et al.*, 1993). Indeed, several components of the yeast actin cytoskeleton are not needed for growth at 24–30°C, but are required for growth at higher temperatures (Welch *et al.*, 1994). In *cdc28-1* and *cdc28-4* mutants, stimulation of SLT2 tyrosine phosphorylation at 37°C is appreciably delayed compared with the wild-type cells. These mutants arrest cell division at 37°C in an unbudded state in the G_1 phase (Hartwell, 1973; Reed, 1980). Interestingly, on incubation at 37°C, these cells begin forming projections, a manifestation of polarized growth, near the time that the level of SLT2 tyrosine phosphorylation is increased. Thus, CDC28 function appears to be required for a timely activation of SLT2, but activation pathways independent of CDC28 also exist.

Yeast cells also carry out polarized growth during mating (Cross *et al.*, 1988). The shmoo is a polarized growth projection that is directed towards the mating partner. SLT2 kinase activation by α-factor is consistent with a role for this pathway in stimulating polarized cell growth during conjugation. α-Factor treatment inhibits CDC28 kinase activity (Wittenberg and Reed, 1988; Tyers *et al.*, 1993; Peter and Herskowitz, 1994) while activating SLT2 tyrosine phosphorylation and kinase activity. Thus, although CDC28 contributes to the timely activation of SLT2 during vegetative cell division, the mating pheromones activate SLT2 through a distinct pathway involving STE20 (see below).

SLT2 activation pathways

Our data and those of others suggest that at least two pathways contribute to SLT2 activation (Figure 8). A common trunk in these pathways involves the MKK1/MKK2 and BCK1(SLK1) kinases (Costigan *et al.*, 1992; Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993; Mazzoni *et al.*, 1993; Kamada *et al.*, 1995). Based on genetic data, and in analogy with other MAP kinase signal transmission modules, MKK1 and MKK2 are thought to be a redundant pair of dual-specificity kinases that activate SLT2 by direct phosphorylation of Thr190 and Tyr192 residues (Lee *et al.*, 1993; Martin *et al.*, 1993). MKK1 and MKK2 would themselves be activated through phosphorylation by BCK1(SLK1) (Irie *et al.*, 1993; Kamada *et al.*, 1995). Support for this scheme comes from our demonstration that SLT2 tyrosine phosphorylation is abolished in a *bck1(slk1)* deletion mutant. We propose

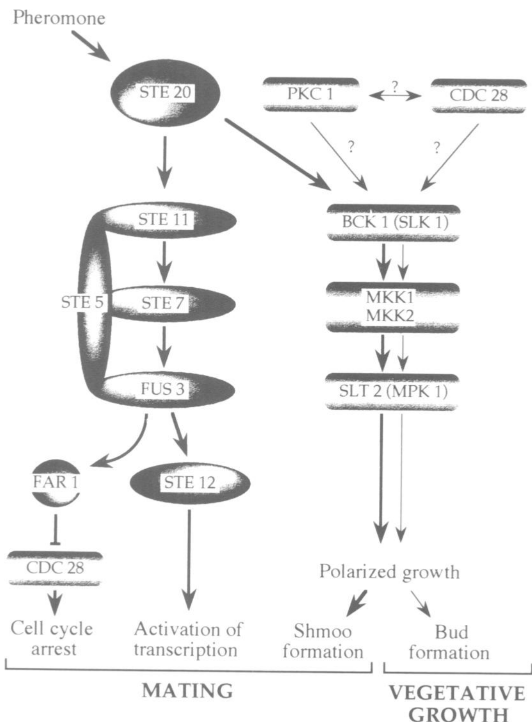

Fig. 8. Model of SLT2 and FUS3 MAP kinase activation pathways.

that upstream of BCK1, activation of the pathway occurs in at least two different ways as a function of the activating stimulus. During vegetative growth, SLT2 tyrosine phosphorylation is inhibited at mitosis and restored in the following cell cycle at the time of bud initiation. Tyrosine phosphorylation of SLT2 is strikingly delayed in a *cdc28* mutant, so we assume that CDC28 activates the SLT2 pathway, although we do not know by what mechanism. Although delayed, SLT2 tyrosine phosphorylation eventually occurs in the absence of CDC28. Thus, an activation pathway independent of CDC28 must exist as well. Such a pathway could involve the yeast protein kinase C (PKC1) that has been proposed to act upstream of BCK1 (Lee and Levin, 1992; Kamada *et al.*, 1995). However, the relationship between PKC1 and CDC28 regarding SLT2 activation remains to be determined.

Phosphorylation of SLT2 on tyrosine is also induced by treatment of yeast cells with the mating pheromone α-factor. α-Factor induces the FUS3/KSS1 MAP kinase pathway, leading to the transcriptional activation of a series of genes involved in conjugation and an arrest of cell division in the G_1 phase caused by inactivation of the CDC28 protein kinase (Cross *et al.*, 1988; Herskowitz, 1995; Schultz *et al.*, 1995). Thus, activation of both MAP kinase pathways by α-factor occurs independently of CDC28. Inactivation of BCK1(SLK1), the presumed MEK kinase of the SLT2 pathway, prevents α-factor from inducing SLT2 tyrosine phosphorylation. Thus, STE11, the MEK kinase of the FUS3/KSS1 pathway, is unable to function in place of BCK1 under these conditions. STE20 is a kinase that has been proposed to activate the FUS3/KSS1 MAP kinase pathway upstream of STE11 in an as yet undefined manner in response to α-factor. We show that STE20 is also required for SLT2 tyrosine phosphorylation induced by α-factor. This induction does not require the STE12 transcription factor which acts downstream of the FUS3/KSS1 MAP kinase in the transcriptional activation of genes involved in mating. We thus suggest that STE20 is a common node at which α-factor activates both MAP kinase pathways. Interestingly, STE20 is also implicated in the activation of MAP kinase pathways required for haploid invasive growth (Roberts and Fink, 1994) and diploid pseudohyphal growth (Gimeno *et al.*, 1992).

Physiological function of the SLT2 pathway

The SLT2 pathway is implicated in yeast cytoskeletal and cell wall integrity, in polarized cell growth, in nutrient response and in response to hypo-osmotic and heat shock (Torres *et al.*, 1991; Costigan *et al.*, 1992, 1994; Irie *et al.*, 1993; Lee *et al.*, 1993; Mazzoni *et al.*, 1993; Costigan and Snyder, 1994; Kamada *et al.*, 1995). Its role in these disparate biological processes raises two questions: first, does activation of the pathway occur by more than one mechanism and second, which targets are modified by the pathway in these different biological processes. SLT2 activation in response to α-factor treatment, heat and hypo-osmotic shock has been proposed to occur as a response to a signal generated by stretching of the plasma membrane. In this light, a single activating stimulus is proposed as a common molecular event underlying the response of the pathway (Kamada *et al.*, 1995; Levin and Errede, 1995). Another possibility is that the SLT2 pathway is activated differentially in order to modify a common target that can influence all of these processes. The yeast actin cytoskeleton is involved in polarized cell growth, cell integrity, response to osmotic shocks and nutrient response (Novick and Botstein, 1985; Johnston *et al.*, 1991; Vojtek *et al.*, 1991; Chowdhury *et al.*, 1992; Liu and Bretscher, 1992; Bauer *et al.*, 1993), the same processes that are affected by the SLT2 pathway. Thus, the actin cytoskeleton may be an important target of the SLT2 pathway activated by diverse stimuli.

Materials and methods

Strains and plasmids

Yeast strains used in this study are congenic with S288C (Sikorski and Hieter, 1989) and are described in Table I.

An HA epitope tag of nine amino acids (YPYDVPDYA) was introduced between Leu468 and Leu469 near the carboxy-terminus (amino acid 484) of SLT2 in the following way. The *Xba*I site within the *SLT2* coding region of pRS316-SLT2 (Mazzoni *et al.*, 1993) was made unique by partially digesting the plasmid DNA with *Xba*I, treating with Klenow DNA polymerase to fill in the 5′ ends, and recircularizing the DNA with T4 DNA ligase. The resulting plasmid was digested with *Xba*I and the linear plasmid was recircularized with T4 DNA ligase in the presence of an excess of two complementary oligonucleotides encoding the HA epitope sequence: 5′-CTAGGCTAGCGTAATCTGGAACATCGTATG-GGTAGC-3′ and 5′-CTAGGCTACCCATACGATGTTCCAGATTACG-CTAGC-3′. Plasmids having incorporated the complementary oligonucleotides had lost the *Xba*I site and gained an *Nhe*I site. DNA sequencing of the *SLT2* gene of one such plasmid, pRS316-SLT2HA, confirmed that a single HA-encoding sequence had been inserted in-frame within the *SLT2* coding sequence. pRS316 is a *URA3* centromeric vector (Sikorski and Hieter, 1989). A 2.2 kb *Kpn*I–*Not*I *SLT2HA* fragment from pRS316-SLT2HA was also cloned into the multicopy pFL44 (2 μm *URA3*) (Bonneaud *et al.*, 1991) and pRS424 (2 μm *TRP1*) (Christianson *et al.*, 1992) vectors by insertion in the *Kpn*I–*Not*I sites of their polylinkers. All three plasmids complemented the temperature-sensitive growth defects of a *slt2::HIS3* mutant.

Site-directed mutagenesis of SLT2HA

The K54R and Y192F mutations were introduced into *SLT2HA* by the PCR-based overlap extension technique (Ho *et al.*, 1989) using pFL44-

Table I. Yeast strains made and used in this study

Name	Genotype
PZY142	*MAT***a**, *ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, ade2-101, bar1::HIS3*/pRS316-SLT2HA
PZY143	*MAT***a**, *ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, ade2-101, bar1::HIS3*/pFL44
PZY144	*MAT***a**, *ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, ade2-101, bar1::HIS3*/pRS316
PZY171	*MATα, cdc28-4, ura3-52, leu2, lys2-801, his3Δ200*/pRS316-SLT2HA
PZY173	*MAT***a**, *cdc28-1, ura3-52, leu2Δ1, lys2-801, his3Δ200, ade2-101*/pRS316-SLT2HA
PZY181	*MAT***a**, *ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, ade2-101, bar1::HIS3*/pFL44-SLT2HA
PZY184	*MAT***a**, *slk1Δ1::TRP1, ura3-52, leu2Δ, lys2-801, his3Δ200, trp1Δ1, ade2-101, bar1::LEU2*/pRS316-SLT2HA
CMY826	*MAT***a**, *ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, ade2-101, bar1::HIS3*
CMY912	*MAT***a**, *ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, ade2-101, bar1::HIS3*/pRS424-SLT2HA
CMY913	*MAT***a**, *ste20::URA3, ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, bar1::HIS3*/pRS424-SLT2HA
CMY914	*MAT***a**, *ste12::LEU2, ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ63, bar1::HIS3*/pRS424-SLT2HA
CMY918	*MAT***a**, *ste11::HIS3, ura3-52, leu2Δ1, lys2-801, his3Δ200, trp1Δ1, bar1::LEU2*/pRS424-SLT2HA
SC55	*MAT***a**/*MATα, ura3-52/ura3-52, leu2Δ1/+, lys2-801/lys2-801, his3Δ200/his3Δ200, trp1Δ1/trp1Δ1, ade2-101/ade2-101*

SLT2HA DNA as the template. An initial series of amplifications was carried out with the *Pfu* proofreading DNA polymerase (Stratagene) using the M13 universal sequencing primer as the upstream oligonucleotide and the sequence 5′-TTGTCACTTTTCTGATGGCAACT-3′ (encoding K54R) or 5′-AGTGGCCACGAACTCCGTCAA-3′ (encoding Y192F) as the downstream primer. These amplified fragments contained pFL44 polylinker sequence, the SLT2 promoter sequence and the 5′ end of the SLT2 coding sequence terminating with the mutant K54R or Y192F sequence at the 3′ extremity. A second series of PCR amplifications employed as upstream primers the complementary sequence of the mutagenic primers, namely 5′-AGTTGCCATCGAAAAGTGACAA-3′ for K54R or 5′-TTGACGGAGTTCGTGGCCACT-3′ for Y192F, and as downstream primer the *SLT2* sequence 5′-CACTACACACAGGTTCG-3′ (oligo G1). These amplifications produced fragments containing the mutant K54R or Y192F sequence at their 5′ extremity and the *SLT2* sequence 5′-CGAACCTGTGTGTAGTG-3′ at their 3′ extremity. Finally, the overlapping mutant fragments were joined by mixing and performing a final PCR amplification in the presence of the M13 universal primer as upstream sequence and oligo G1 as the downstream primer. The amplified fragments were digested with *Not*I + *Sac*I, and the resulting 800 bp mutant DNA fragments were purified from agarose gels and substituted for the corresponding wild-type DNA fragment contained on the pFL44-SLT2HA plasmid. Incorporation of the mutations was verified by DNA sequencing.

Immunoprecipitation of SLT2HA and determination of its phosphotyrosine content

Yeast cells were grown to log phase in synthetic complete media while maintaining selection for *URA3* or *TRP1* plasmids at 30°C for the wild-type and at 24°C for the ts mutants. Cells were then diluted to OD_{600} = 0.25 in YPD and allowed to grow one generation before cells were temperature-shifted or treated with 1 μM α-factor as described in the figure legends. An equal volume of ice was then added to 25 ml of cells in a 50 ml Falcon centrifuge tube. Cells were pelleted and transferred to an Eppendorf tube in 150 μl of cold lysis buffer [50 mM Tris–HCl (pH 7.5), 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 5 mM Na pyrophosphate, 2 μg/ml each of leupeptin, aprotinin, chymostatin and pepstatin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM β-glycerophosphate, 15 mM p-NO_2-phenylphosphate, 10 mM Na orthovanadate]. An equal volume of glass beads was added and cells were lysed by vortexing. Extracts were then clarified by 15 min of centrifugation at 4°C. The supernatant was collected and incubated at 4°C for 2 h with 30 μl of protein A–Sepharose beads containing covalently coupled mouse monoclonal 12CA5 anti-HA immunoglobulin (Field *et al.*, 1990). Saturating quantities of 12CA5 antibodies were coupled to the protein A–Sepharose as described (Harlow and Lane, 1988). Beads were washed twice with lysis buffer. Laemmli buffer (25 μl) was added, proteins were denatured by heating at 90°C for 4 min and then separated on SDS–8% polyacrylamide gels. Ten μl of each sample was used for anti-phosphotyrosine Western blots and 2 μl for anti-HA Western blots. Proteins were transferred to Immobilon-P membranes (Millipore Corp.) and membranes were incubated with either purified 4G10 mouse monoclonal anti-phosphotyrosine IgG (Upstate Biotechnology Incorporated) at a concentration of 2 μg/ml or with a 1/1000 dilution of 12CA5 anti-HA ascites fluid. Primary antibodies were revealed by enhanced chemiluminescence (Amersham ECL) using a horseradish peroxidase (HRP)-conjugated anti-mouse antibody. In these Western blots, tyrosine-phosphorylated SLT2HA migrates with an apparent M_r of 62 kDa at a position slightly slower than the 12CA5 IgG heavy chain. Note that even after covalent coupling of most of the anti-HA antibodies to the protein A–Sepharose, the small amount of heavy chain released upon boiling in SDS sample buffer produced a strong signal after reaction with the HRP-conjugated anti-mouse antibody.

Kinase activity of SLT2HA immunoprecipitates

Preparation of protein extracts and SLT2HA immunoprecipitation was performed as described above, except that the lysis buffer was composed of 25 mM Tris–HCl (pH 7.5), 5% glycerol, 10 mM EGTA, 10 mM Mg Cl_2, 1 mM dithiothreitol (DTT), 1 mM Na N_3, 2 μg/ml of leupeptin, aprotinin, chymostatin and pepstatin, 1 mM PMSF, 50 mM β-glycerophosphate, 15 mM p-NO_2-phenylphosphate and 10 mM Na orthovanadate. Moreover, most of the immunoprecipitations were carried out by the sequential addition of 3 μl of anti-HA 12CA5 ascites fluid with a 1 h incubation at 4°C followed by the addition of 30 μl of protein A–Sepharose with a further incubation for 1 h at 4°C. Immunoprecipitates were washed twice with lysis buffer, twice with lysis buffer containing 100 mM NaCl + 0.1% Triton X-100 and twice with kinase buffer [20 mM Tris-HCl (pH 7.5), 7.5 mM $MgCl_2$]. Phosphorylation of endogenous, associated substrates was initiated by the addition of 15 μl of kinase buffer containing 10 μM ATP and 5 μCi [γ-^{32}P]ATP to the SLT2HA immunoprecipitate. The MBP kinase reaction contained, in addition, 10 μg of MBP (Sigma). Kinase reactions were carried out at 30°C for 15 min and stopped by the addition of SDS sample buffer and heating at 90°C for 4 min. For double immunoprecipitation experiments, the immune complex kinase reactions were carried out as described above, except that the reaction was terminated by the addition of an equal volume of 2% SDS and heating at 90°C for 5 min to denature the protein complexes. The reactions were then diluted 10-fold with immunoprecipitation buffer, and proteins containing the HA tag were reprecipitated by the sequential addition of anti-HA antibodies and protein A–Sepharose beads.

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