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Spa2p Functions as a Scaffold-like Protein to Recruit the Mpk1p MAP Kinase Module to Sites of Polarized Growth

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Summary

Scaffold proteins play a major role in regulating MAP kinase pathways. In yeast, the Mpk1p-MAP kinase pathway functions to maintain the integrity of the cytoskeleton and the cell wall [1]. In this module, the MEKK Bck1p functions upstream of the MEKs Mkk1p and Mkk2p, which in turn activate the MAP kinase Mpk1p. Mpk1p regulates several nuclear targets, including the transcription factors Rlm1p and SBF, and the two HMG1-like proteins NHP6A and NHP6B. Here we show that Mpk1p constitutively shuttles between the nucleus and the cytoplasm, and both Mpk1p and Mkk1p localize to sites of polarized growth in a Spa2p-dependent manner. Spa2p belongs to a group of proteins that includes Bni1p, Bud6p, and Pea2p, which are involved in the dynamic organization of the actin cytoskeleton during polarized growth [2]. FRAP analysis shows that Spa2p-GFP is stably anchored at bud tips, whereas Mpk1p binds transiently. Spa2p interacts with Mkk1p and Mpk1p, and membrane bound Spa2p is sufficient to recruit Mkk1p and Mpk1p but not other MAP kinases to the cell cortex. Taken together, these results suggest that Spa2p functions as a scaffold-like protein for the cell wall integrity pathway during polarized growth.

Results

Mpk1p-GFP and Mkk1p-GFP Accumulate at Sites of Polarized Growth

The Mpk1p MAP kinase pathway assures cell integrity and prevents cell lysis during budding and mating [1]. The Mpk1p module consists of the MEKK Bck1p and the two functionally redundant MEKs Mkk1p and Mkk2p, which in turn activate the MAP kinase Mpk1p (also known as Slt2p) [3]. Bck1p functions downstream of Rho1p and Pkc1p [4], but it is also activated by the pheromone response pathway in a PKC-independent manner [5]. To determine the subcellular localization of Mpk1p, Mkk1p, Mkk2p, and Bck1p, we fused the green fluorescent protein (GFP) to the carboxyl terminus and localized the functional fusion proteins in wild-type cells after expression from the endogenous (Mpk1p-GFP) or the *ADH1* promoter. Mpk1p-GFP was predominantly nuclear at all stages of the cell cycle (Figure 1A), whereas Mkk1p-GFP and Mkk2p-GFP were dispersed throughout the cytoplasm. Interestingly, all three proteins localized to the tip of small buds and accumulated at the mother-bud region late in mitosis. In addition, Mpk1p-GFP, Mkk1p-GFP, and Mkk2p-GFP were found at tips of mating projections (Figure 1B). In contrast, Bck1p-GFP was cytoplasmic at all cell cycle stages, and no accumulation at sites of polarized growth was apparent (our unpublished data).

Spa2p Is Required for the Accumulation of Mpk1p and Mkk1p at Sites of Polarized Growth

The localization of Mpk1p and Mkk1p is reminiscent of Spa2p, which is part of a complex that regulates the dynamic organization of the actin cytoskeleton at sites of polarized growth [2]. Indeed, like Spa2p (Figure 1; [6]), Mpk1p-GFP and Mkk1p-GFP were recruited to the incipient bud site in the presence of latrunculin-A (Lat-A), indicating that actin polarization is not required for their localization. Importantly, both Mpk1p-GFP and Mkk1p-GFP failed to accumulate at bud tips and the bud neck region in *spa2*Δ or *spa2-2* cells (Figure 2A). In contrast, Mpk1p was not necessary for localizing Spa2p-GFP or Mkk1p-GFP (Figure 2B). Mpk1p-GFP localized normally in *pea2*Δ or *pea2-2* cells, whereas surprisingly, Mkk1p-GFP was barely detected at bud tips (Figure 2C and our unpublished data), although it accumulated at the bud neck region. Finally, Mpk1p-GFP and Mkk1p-GFP localized efficiently to bud tips in *bni1*Δ and *bni1-10* cells (Figure 2D and our unpublished data), demonstrating that the apical growth defect of *spa2*Δ cells does not solely account for its defective localization of the Mpk1p module. These results suggest that Spa2p is required for localizing the Mpk1p MAP kinase module to sites of polarized growth.

A Transmembrane Fusion of Spa2p Is Able to Recruit Mkk1p-GFP and Mpk1p-GFP, but not Fus3p-GFP, to the Plasma Membrane

Spa2p has been shown previously to interact with Mkk1p and Ste7p [7]. We used two-hybrid assays to examine whether Spa2p also binds Mpk1p. Indeed, Spa2p interacted with Mpk1p but not with Fus3p or Kss1p (Figure 3A and our unpublished data). Conversely, Fus3p but not Mpk1p interacted with the scaffold Ste5p, which recruits Fus3p to shmoo tips in response to pheromones. Interestingly, Mpk1p-GFP was undetectable at tips of the peanut-like mating projections of *spa2-2* cells, whereas Fus3p-GFP localized normally (Figure 3B). Taken together, these results suggest that Spa2p specifically localizes Mpk1p but does not affect other MAP kinases.

To determine whether binding of Mkk1p-GFP and Mpk1p-GFP to Spa2p may be responsible for their re-

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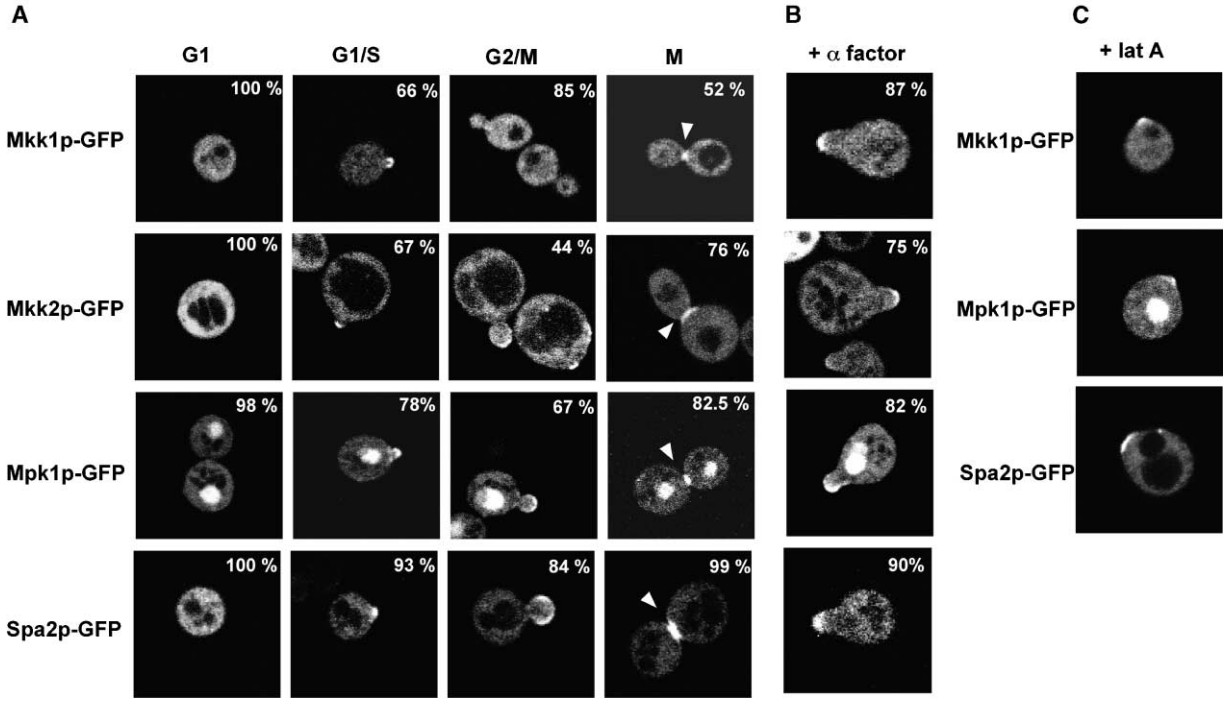

Figure 1. The Localization of Mpk1p-GFP, Mkk1p-GFP, and Spa2p-GFP during the Cell Cycle and in Response to Pheromones

Wild-type cells (YFD126) expressing the indicated GFP-fusion proteins from the endogenous (Mpk1p-GFP) or the *ADH1* promoter were grown at 30°C until mid-log phase and analyzed by GFP microscopy. Cells at different stages of the cell cycle are shown in panel (A), and panel (B) shows cells that were treated with α-factor for 2 hr. The arrowheads point to the mother-bud neck region in mitotic cells. The numbers indicate the percentage (%) of cells with the GFP fusion protein located at the indicated sites of polarized growth; at least 200 cells were counted. (C) Wild-type cells were arrested in early G1 by a nutritional block [18] and were released into rich media containing 20 μg/ml Lat-A. The localization of the indicated GFP fusion proteins was analyzed after 45 min, which corresponds to the time of bud emergence in control cells not treated with Lat-A. Note that recruitment of Mpk1p-GFP and Mkk1p-GFP to the incipient bud site is independent of an intact actin cytoskeleton.

cruitment to the plasma membrane, we fused Spa2p to the transmembrane domain of Snc2p (Spa2p-CTM) [8]. Although Spa2p-CTM is expected to localize uniformly to the plasma membrane (see below), this construct was not sufficient to constitutively activate Mpk1p as assayed by immunoblotting for phosphorylated Swi6p (our unpublished data) [9]. Interestingly, expression of Spa2p-CTM in wild-type cells was able to recruit Mkk1p-GFP and Mpk1p-GFP but not Fus3p-GFP or Ste7p-GFP to the plasma membrane (Figure 3C), suggesting that Spa2p is not only required but also sufficient to target Mpk1p and Mkk1p to the cell cortex. In contrast, Ste5p-CTM failed to localize Mpk1p and Mkk1p at the cell cortex, although it readily recruited Ste7p-GFP or Fus3p-GFP (Figure 3D) [10]. No membrane recruitment of Mpk1p-GFP was detected without induction of Spa2p-CTM or after expression of Spa2p lacking the CTM-domain (our unpublished data). Taken together, these data suggest that Spa2p is necessary and sufficient for specific membrane recruitment of Mpk1p and Mkk1p.

Mpk1p Rapidly Dissociates from Spa2p and Translocates into the Nucleus

We used fluorescence recovery after photobleaching (FRAP, [11]) to examine whether Mpk1p-GFP shuttles between the nucleus and sites of polarized growth. The nucleus of wild-type cells expressing Mpk1p-GFP was photobleached as indicated in Figure 4A, and the recovery of nuclear fluorescence was quantified and plotted against the time (in seconds) after photobleaching. For the control, nuclear fluorescence of Mpk1p-GFP was measured in cells, which were not photobleached (squares). Interestingly, nuclear fluorescence of Mpk1p-GFP after photobleaching was rapidly restored (Figure 4A), with a half-life of recovery ($\tau_{1/2}$) of 1.5 ± 0.5 s. Exposing the cells to high salt or increased temperature to activate Mpk1p did not alter this rapid nuclear/cytoplasmic exchange rate (our unpublished data), suggesting that the activity of Mpk1p does not regulate its nuclear shuttling. We also used FRAP to measure the turnover kinetics of Mpk1p and Spa2p at sites of polarization (Figure 4B). Whereas Mpk1p-GFP at tips of small buds recovered within a fraction of a second ($\tau_{1/2}$ = 0.6 ± 0.1 s), Spa2p-GFP remained there significantly longer ($\tau_{1/2}$ = 6.7 ± 0.8 s), implying that Mpk1p may rapidly dissociate from Spa2p at sites of polarized growth. Finally, we used FLIP (fluorescence loss in photobleaching) to determine whether membrane-associated Mpk1p-GFP dissociates from Spa2p-CTM and translocates into the nucleus. In these experiments, we repeatedly photobleached Mpk1p-GFP at the plasma membrane but monitored GFP-fluorescence in the nucleus (Figure 4C). Indeed, nuclear fluorescence decreased

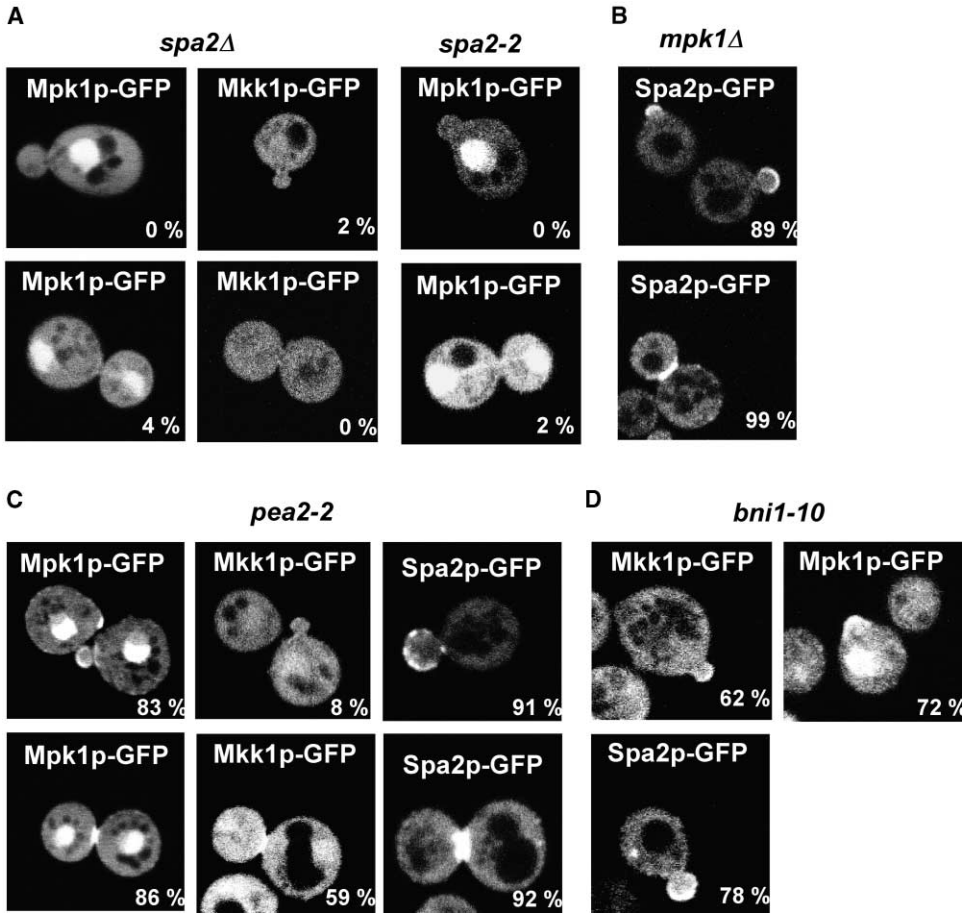

Figure 2. The Accumulation of Mpk1p-GFP and Mkk1p-GFP at Sites of Polarized Growth Is Dependent on Spa2p

The localization of Mpk1p-GFP, Mkk1p-GFP, and Spa2p-GFP was analyzed by fluorescence microscopy as indicated in *spa2*Δ (YFD240) and *spa2-2* (YFD239) cells (A), as well as *mpk1*Δ (YFD258 [B]), *pea2−2* (YFD257 [C]) and *bni1-10* (YFD259, [D]) cells grown at 25°C until mid-log phase. The numbers indicate the percentage (%) of cells with the GFP fusion protein located at bud tips or the mother-bud region, respectively. Note that the accumulation of Mpk1p-GFP and Mkk1p-GFP at sites of polarized growth requires functional Spa2p.

shortly after photobleaching, implying that Mpk1p-GFP must exchange between the plasma membrane and the nucleus. We conclude that Mpk1p only transiently associates with Spa2p at sites of polarization.

Discussion

Mpk1p and Mkk1p Accumulate at Sites of Polarized Growth

Several lines of evidence suggest that Spa2p functions as a scaffold-like molecule to recruit the Mpk1p-MAP kinase module to sites of polarized growth. First, Mkk1p [7] and Mpk1p specifically interact with Spa2p. Second, Spa2p, Mkk1p, and Mpk1p accumulate at sites of polarized growth in an actin-independent manner. Third, Mpk1p and Mkk1p fail to accumulate at bud tips and mating projections in *spa2*Δ cells. Finally, Spa2p-CTM is sufficient to recruit Mkk1p and Mpk1p to the cell cortex. Mkk1p interacts with the amino-terminal domain of Spa2p, which is not required for its localization [12]. Likewise, Spa2p is efficiently localized to sites of polarized growth in *mpk1*Δ cells, suggesting that the localization of Spa2p precedes the recruitment of the Mpk1p-MAP kinase module. Based on these results, we propose that Spa2p provides a platform for Mpk1p activation at sites of polarized growth, whereas active Mpk1p dissociates from Spa2p and rapidly translocates into the nucleus to phosphorylate downstream substrates. However, Mpk1p is activated efficiently in *spa2*Δ cells exposed to high salt or heat-shock conditions [7, 13], demonstrating that Spa2p is not essential for Mpk1p activation under stress conditions that affect the entire cell cortex. Rather than generally activating Mpk1p, Spa2p may thus be involved in concentrating Mpk1p activity to specific regions on the cell cortex, in particular to sites of polarized growth.

Coordination of Cellular Responses at the Site of Polarized Growth

Polarized growth is regulated by local activation of Cdc42p and Rho1p [14], which through distinct effectors trigger assembly of the actin cytoskeleton, increased cell wall synthesis, and activation of the Mpk1p MAP kinase cascade. For example, Rho1p activates 1,3-β-glucan synthase [15], the enzyme that catalyzes the local synthesis of the major structural component of the

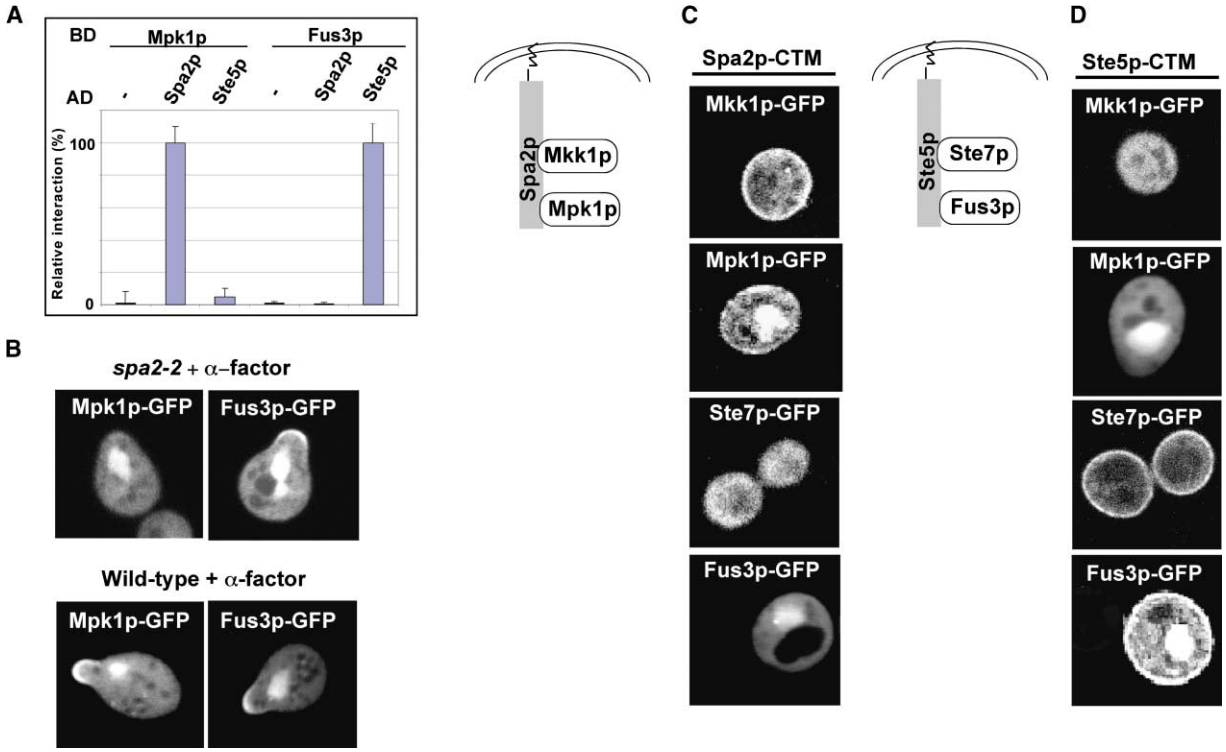

Figure 3. Spa2p Binds Specifically to Mkk1p and Mpk1p

(A) The interaction between the MAP kinases Mpk1p and Fus3p fused to the DNA binding domain (BD) with Spa2p and the scaffold protein Ste5p fused to the activation domain (AD) was determined by a two-hybrid assay. The interactions were quantified and represented as a percentage (%) of total Miller Units with standard deviations.

(B) Mpk1p-GFP and Fus3p-GFP were expressed in wild-type or *spa2-2* cells from the *GAL* promoter, and their localization was analyzed by fluorescence microscopy after cells were treated with α-factor for 2 hr. Note that Fus3p-GFP but not Mpk1p-GFP is able to accumulate at shmoo tips in *spa2-2* cells.

(C and D) The localization of Mkk1p-GFP, Mpk1p-GFP, Ste7p-GFP, and Fus3p-GFP was analyzed by GFP microscopy in wild-type cells (YFD126) expressing either Spa2p-CTM (left panel) or Ste5p-CTM (right panel). Note that Spa2p-CTM is able to recruit components of the cell integrity pathway to the cell cortex.

cell wall. Activated Rho1p also interacts with Pkc1p [16, 17], which phosphorylates Bck1p to activate the kinase cascade. Interestingly, Cdc42p is required to recruit Spa2p [18], which through its proposed scaffolding role may in turn place Bck1p in close proximity to Pkc1p. We speculate that membrane bound Spa2p (Spa2p-CTM) is not sufficient for activating Mpk1p in vivo because the activation of Bck1p still requires activated Rho1p. Thus, two independent pathways may cooperate to activate the Mpk1p MAP kinase module at sites of polarized growth. Such a dual mode of MAP kinase activation may prevent inappropriate MAP kinase signaling and influence the kinetics of MAP kinase activation in vivo.

Role of Scaffold Molecules in MAP Kinase Signaling

The best-studied scaffold protein involved in MAP kinase signal transduction is Ste5p [19], which is required to activate Fus3p during mating. When one compares the roles of Ste5p and Spa2p, some common schemes emerge for the function of scaffolds in MAP kinase signaling. Both Ste5p and Spa2p recruit specific MAP kinase modules to sites at the cell cortex; these sites are determined by internal cues or extracellular signals. Available evidence suggests that these cortical sites represent stable sites of MAP kinase activation, whereas activated MAP kinases dissociate and phosphorylate targets predominantly localized in the nucleus. At least in the case of Ste5p, activation of Fus3p may increase its dissociation from Ste5p and thereby promote rapid nuclear import of active MAP kinase [10]. Thus, the subcellular localization of these MAP kinases is dynamic; they are activated at the cell cortex by binding to scaffold proteins, whereas they function at least in part in the cell nucleus by adapting transcription to the environmental conditions. From all yeast MAP kinases, only Hog1p appears to be regulated at the level of nuclear transport [20]. Like Mpk1p, the MAP kinases Fus3p and Kss1p shuttle between the nucleus and the cytoplasm even in the absence of activation [21]. Thus, although all MAP kinases transmit signals from the plasma membrane to the nucleus, regulation of nuclear transport may not be a common mechanism for controling MAP kinase pathways.

Conclusions

In this study, we show that Mpk1p and Mkk1p accumulate at sites of polarized growth during budding and in response to mating pheromones. Spa2p is necessary and sufficient for this localization, and Spa2p interacts

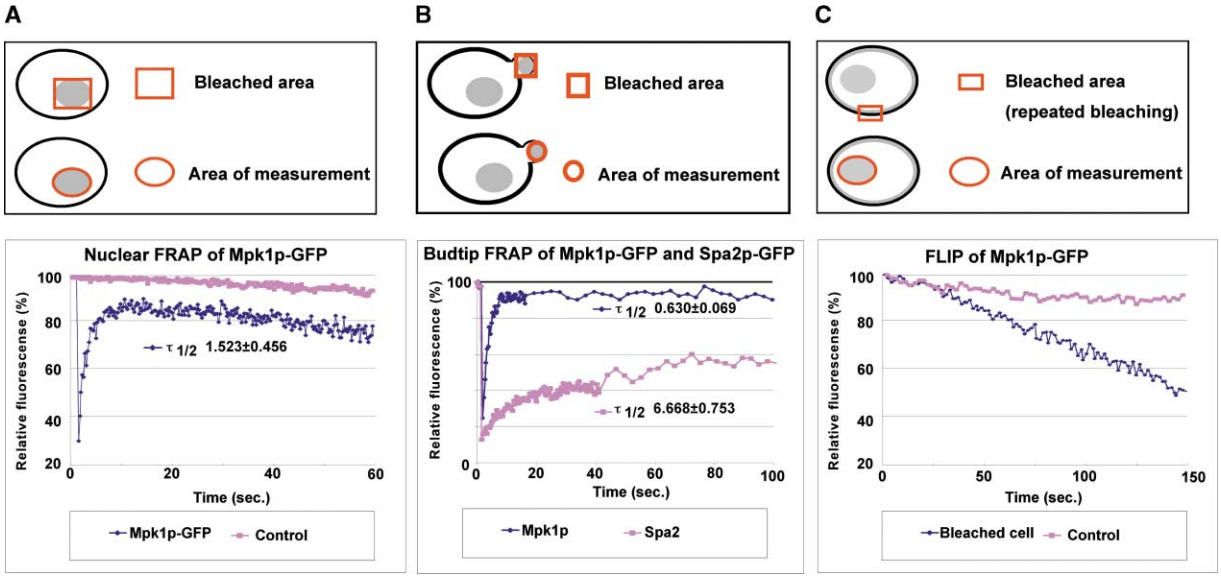

Figure 4. Mpk1p-GFP Transiently Binds to Spa2p at the Plasma Membrane

(A) Nuclear recovery of Mpk1p-GFP (diamonds) was measured by FRAP in wild-type cells (YFD126), as schematically indicated in the upper panel. Nuclear recovery was quantified and shown as $\tau_{1/2}$ with standard deviations. An unbleached nucleus was included as a control (squares).
(B) The turnover of Mpk1p-GFP (diamonds) and Spa2p-GFP (squares) at the tips of small buds was determined as outlined in the upper panel. The recovery was quantified and shown as $\tau_{1/2}$ with standard deviations.
(C) The translocation of Mpk1p-GFP from the plasma membrane (PM) to the nucleus was determined by FLIP as depicted in the upper panel. A segment of the PM of wild-type cells expressing both Mpk1p-GFP and Spa2p-CTM was repeatedly photobleached, and the decrease of nuclear GFP fluorescence was measured in a time-dependent manner (diamonds). As a control, an identical area in the cytoplasm was bleached (squares).

specifically with Mpk1p. Using FRAP and FLIP assays, we demonstrate that Mpk1p shuttles between the nucleus and the site of polarization. Based on these results, we propose that Spa2p functions as a scaffold-like protein to localize the Mpk1p MAP kinase module at sites of polarized growth.

Experimental Procedures

Strains, Media, and Genetic Techniques

Yeast strains are described in Table 1. α-factor (synthesized by Lipal Biochemicals, Zürich, Switzerland) was added to a final concentration of 5 μg/ml (2 mg/ml stock solution in 10 mM HCl).

Plasmid Constructions

Plasmids and oligonucleotides (synthesized by Genset, Paris, France) are listed in Table 2. The CTM sequence (WWKDLKMRMC LFLVVIILLVVIIVPIVVHFS) was fused to the amino terminal domain of Spa2p, as described previously for Ste5p [8].

Microscopy and FRAP and FLIP Experiments

GFP-tagged proteins were visualized by confocal microscopy or on a Zeiss Axiovert 100 with a Chroma GFP II filter, recorded with a Photometrics CCD camera, and analyzed with IPlab Spectrum software. G0-synchronization experiments were carried out as described [18]. For induction of protein expression from the *GAL1,10*-promoter (FRAP and FLIP experiments), cells were grown at 30°C to early log phase in medium containing 2% raffinose, at which time 2% galactose was added for 3 hr. Strains expressing temperature-sensitive (ts) alleles were grown at 25°C and shifted to 37°C for 2 hr. Where indicated, cycloheximide (CHX; Sigma) was added to a final concentration of 50 μg/ml (stock solution 10 mg/ml in H_2O), and cultures were incubated for 1 hr. For the control, cells were fixed with 3.7% formaldehyde (stock solution 37%; Merck) for 45 min before FRAP analysis. FRAP and FLIP analysis was performed with a Zeiss LSM510 confocal laser-scanning microscope, with a LP505 nm filter, essentially as described [10].

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