The function of two closely related Rho proteins is determined by an atypical switch I region

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Summary
We show here that the encoded proteins of the two duplicated RH01 genes from the filamentous fungus Ashbya gossypii, AgRH01a and AgRH01b have functionally diverged by unusual mutation of the conserved switch I region. Interaction studies and in vitro assays suggest that a different regulation by the two GTPase activating proteins (GAPs) AgLrg1 and AgSac7 contributes to the functional differences. GAP-specificity and protein function is determined to a large part by a single position in the switch I region of the two Rho1 proteins. In AgRho1b, this residue is a tyrosine that is conserved among the Rho-protein family, whereas AgRho1a carries an atypical histidine at the same position. Mutation of this histidine to a tyrosine changes GAP-specificity, protein function and localization of AgRho1a. Furthermore, it enables the mutated allele to complement the lethality of an AgRH01b deletion. In summary, our findings show that a simple mutation in the switch I region of a GTP-binding protein can change its affinity towards its GAPs, which finally leads to a decoupling of very similar protein function without impairing effector interaction.

Introduction
Small GTP-binding proteins are a ubiquitous protein class in eukaryotic cells. They are involved in diverse processes such as cancer development, cell morphology or vesicle trafficking (Takai et al., 2001). The structure and function of this protein class are highly conserved and well understood. Activation of small GTP-binding proteins is characterized by a GDP-GTP exchange involving regulators called guanine nucleotide exchange factors (GEFs). The active, GTP-bound form of the GTP-binding protein binds and activates the effector proteins. Inactivation is mediated by the binding of a GTPase-activating protein (GAP) that increases the intrinsically low GTPase activity of the GTP-binding protein. The large variety of Rho proteins has evolved by mutations that occurred after gene duplication (Boureux et al., 2007). Although the GTPase-switch mechanism remained highly conserved, the interaction interface varies between the different Rho proteins and thereby creates novel signaling junctions (Wherlock and Mellor, 2002). In this study, we have used a comparative analysis of duplicated and non-duplicated Rho proteins to understand how Rho protein function may have diverged after gene duplication.

Yeasts provide an excellent tool for studying gene duplication (Wolfe, 2006). Comparison of the well-annotated genome of Saccharomyces cerevisiae with several complete genomes of other yeasts allows the reliable identification of duplicated genes. The filamentous growing Ashbya gossypii is of special interest for studies on evolution of gene function in combination with the unicellular budding yeast S. cerevisiae (Philippsen et al., 2005). The different growth styles of the two organisms increase the possibility of observing evolutionary changes in function of gene-products that are involved in elementary processes such as polar growth, cell division and cell cycle control.

A typical member of the Rho GTP-binding protein family is the Rho1 protein from S. cerevisiae. Its function and its regulation are complex but well understood (Levin, 2005). Deletion of the RH01 gene is lethal (Madaule et al., 1987), which reflects the multitude of tasks that have been attributed to the encoded protein. Two major functions of RH01, regulation of cell wall biosynthesis and regulation of the actin cytoskeleton, are reflected by two different complementation groups of rho1 mutants (Saka et al., 2001). Cell wall biosynthesis is regulated by interaction of Rho1 with the glucan-synthase complex (Drgonova et al., 1996; Mazur and Baginsky, 1996; Qadota et al., 1996), which produces β-(1,3)-glucan, the major component of the yeast cell wall. Initially, Rho1 was isolated as the regulatory subunit of this complex. Regulation of the actin cytoskeleton is achieved by interaction with two different effector-proteins: the formin Bni1 (Kohno et al., 1996) and the single S. cerevisiae protein kinase C homolog Pkc1 (Nonaka et al., 1995). Although the connection to actin regulation via formins is direct as proteins of this class catalyze polymerization of actin cables, actin regulation via Pkc1 is not well understood and the targets of Pkc1 in actin regulation are unknown. In addition to these two main functions of Rho1, there is evidence for a variety of other functions (reviewed by Levin, 2005).

A first homolog of the S. cerevisiae RHO1 was cloned from A. gossypii before the second copy was discovered during the genome-sequencing project (Wendland and Philippsen, 2001). Deletion mutants of AgRHO1 (open reading frame ABR183W, AgRHO1b) grew slowly, suffered from significant cell lysis and actin defects, and died within 4 days. This suggested functions similar to the S. cerevisiae Rho1. A second RHO1-homolog (ABR182W, AgRHO1a), directly upstream of AgRHO1, was identified after the genome sequence became available (Dietrich et al., 2004). The genomic
organization of the AgRHO1 genes together with the neighboring genes in A. gossypii and S. cerevisiae is shown in Fig. 1A. The two copies very probably originate from a tandem-duplication event. Little is known about the function of AgRho1a and AgRho1b genes in A. gossypii and compared them with their single homolog in budding yeast. Surprisingly, we found that a single residue in AgRho1a is altered in the highly preserved switch I region. This residue contributes to divergence of protein function and alters GAP specificity without impairing effector interaction.

Results

The lytic phenotype of Agrho1aΔ is temperature-dependent

We first characterized Rh1 protein function in A. gossypii to test whether protein function diverged. An alignment of the protein sequences shows that AgRho1a is 73.9% identical to AgRho1b and 75.6% identical to ScRho1, whereas the latter two proteins share the highest number of identical residues (83.3%) (Fig. 1B). As a first step towards defining the functional difference between AgRHO1a and AgRHO1b, we compared the phenotypes of deletion mutants. Although Agrho1aΔ formed a mature mycelium that grew slightly slower than the wild-type control, deletion of AgRHO1b resulted in cells that never reached the state of a mature mycelium and died because of cell lysis (Fig. 1C). Agrho1aΔ cells also lysed but only few hyphae were affected. Fig. 1D shows an example of lysing hyphae taken from Movie 1 (supplementary material). We also monitored the strain deleted for AgRHO1a at different growth temperatures. Lysis of adult mycelia was quantified by an assay that visualizes the release of cytoplasmic alkaline phosphatase upon lysis by a color reaction (Fig. 1E, for details, refer to Materials and Methods). An Agslt2Δ strain was used as a lysis control. AgSLT2 is the homolog of the S. cerevisiae MAP kinase responsible for maintenance of cellular integrity (Torres et al., 1991). In contrast to Agrho1b deletion mutants, Agslt2 deletions can form a mature mycelium despite extensive lysis. Hyphae at the colony border grow normally and cannot be distinguished microscopically from wild type but the inner, older parts of the mycelium show extensive lysis. At 25°C and 30°C, Agrho1a deletion mutants released about half as much alkaline phosphatase as the Agrho1aΔ deletion mutants. Surprisingly, at 37°C, lysis of Agrho1aΔ decreased to about 4% of that of Agslt2Δ cells. As Rho-type GTPases are often regulators of the actin cytoskeleton (Boureux et al., 2007), we speculated that lysis of the Agrho1aΔ might be due to a defect in actin regulation. However, we did not observe significant differences between the actin cytoskeleton of wild-type and Agrho1aΔ cells stained with Alexa-phalloidin (Fig. 2A). As visualization of actin requires fixation of the cells, differences in actin dynamics might have been missed. Actin cables can be visualized in living cells by the Abp140-GFP fusion protein (Yang and Pon, 2002). Interestingly, an Agrho1aΔ strain that carried a plasmid encoding AgAbp140-GFP grew poorly compared with the wild type with the same plasmid (Fig. 2B). Microscopy of the actin cytoskeleton was impossible because the small sick cells had high background fluorescence. However, the phenotype associated with expression of AgABP140-GFP suggests that the actin cytoskeleton of the Agrho1aΔ strain has a weak defect. Therefore, we tested wild type and Agrho1aΔ for sensitivity to the actin polymerization inhibitor Latrunculin A. Fig. 2C,D shows that Agrho1aΔ cells are indeed slightly more sensitive to actin perturbation than wild-type cells, indicated by ~1.5-fold increase of the inhibition zone by 10 nmol Latrunculin A. This suggests an involvement of AgRho1a in actin regulation. The observed lysis of hyphae could therefore be an indirect consequence of the actin defects, whereas the lethal lysis phenotype of the Agrho1b deletion could be the direct consequence of defects in cell wall biosynthesis.

Different localization patterns of AgRho1a and AgRho1b

A difference in protein function may be reflected by a different localization pattern. Therefore, we localized fusions of both proteins...
to YFP (AgRho1a) or CFP (AgRho1b) simultaneously by fluorescence microscopy. As we observed temperature-dependent lysis for the Agrho1aΔ mutants, we speculated that temperature might be an important factor for the function of AgRho1a. To test this, cells were grown either at 25°C or at 37°C. Fig. 3A shows that, at 25°C, AgRho1b localizes predominantly to the cortex and the cytoplasm at the tip of the growing hyphae, whereas AgRho1a localizes to the entire cytoplasm. Interestingly, a 1-hour heat shock at 37°C led to a change of localization pattern for both proteins. Now they were found at the cortex of the entire hyphae. In contrast to the localization of AgRho1b at 25°C, membrane association is not restricted to the tip region of the hyphae. This implies that at higher temperatures both proteins might share more functions than at lower temperatures, which might explain the reduced lysis at higher temperature observed for Agrho1aΔ mutants.

Whereas localization of the two AgRho1-proteins differs at the tip region of hyphae, we found both at developing (Fig. 3B, first row) and mature septa (Fig. 3B, second row), suggesting that both function in septum formation.

To test whether the area of AgRho1b localization at the tip at 25°C overlaps with the area of cell wall biosynthesis, we observed a GFP-fusion to AgRho1b in Aniline Blue stained hyphae. Aniline Blue is incorporated with 1,3-β-glucan into the forming cell wall (Kippert and Lloyd, 1995). Therefore, short incubation times with Aniline Blue preferentially stain the sites of cell wall biosynthesis. As shown in Fig. 3C, an overlap of Aniline Blue staining and GFP-Agrho1b is observed in growing tips and newly emerging branches. Even though this is not proof, a role of AgRho1b in cell wall synthesis is possible.

**Agrho1a does not function in glucan biosynthesis**

We performed complementation assays of yeast mutants to get further insight into the specific functions of the two AgRho1-proteins. *S. cerevisiae* is a close relative of *A. gossypii* but has only a single RHO1 gene. Therefore, complementation of *S. cerevisiae rho1* mutants can be used to assay how the function of duplicated genes from *A. gossypii* may have evolved. To avoid artifacts from heterologous promoters, we constructed *S. cerevisiae* strains with an ORF replacement of the RHO1 gene by each of the *A. gossypii* homologs, resulting in a fusion of the *A. gossypii* genes to the ScRHO1 promoter. Fig. 4A shows representative examples of a tetrad analysis of heterozygous strains carrying ScRHO1 and either AgRho1a (left side) or AgRho1b (right side). Out of 50 tetrads analyzed for AgRHO1a, all showed a 2:2 segregation for viability and none of the viable spores carried the marker for Agrho1aΔ, proving that Agrho1a is unable to complement the loss of ScRHO1. By contrast, segregants of a strain with AgRHO1b replacing ScRHO1 were viable, even though a 2:2 segregation with larger and smaller colonies was observed. The small colonies carried the marker of AgRHO1b in all cases tested. In addition to the tetrad analysis, we tested whether haploid cells, in which the genomic copy of ScRHO1 was replaced by either one of the AgRHO1 alleles, could be forced to loose a plasmid with an *URA3* marker carrying the ScRHO1 gene on medium with 5-Fluoroorotic Acid (Fig. 4B). Consistent with the results from the tetrad analysis, plasmid loss was possible for Scrho1Δ::AgRHO1b but not for Scrho1Δ::AgRHO1a. However, in contrast to the tetrad analysis, where Scrho1Δ::AgRHO1b segregants were smaller, the Scrho1Δ::AgRHO1b strain grew like the wild type suggesting that the reason for the small size of the segregants is delayed germination and not a growth defect. To verify this, we tested the growth of the Scrho1Δ::AgRHO1b strain at high temperature (Fig. 4C), where a potential growth defect should become apparent. However, the segregants behaved like the wild type, even at temperatures as high as 39°C, suggesting that there is no growth defect associated with the Scrho1::AgRHO1b mutant.

This complementation of a Scrho1 deletion suggests that AgRHO1b and ScRHO1 functions are conserved, whereas the
functions of AgRHO1a and ScRHO1 have diverged. The functions of AgRHO1b and ScRHO1 probably reflect the functions of the common ancestor RHOL gene. This raises the question, has AgRHO1a lost the capability to act in both or only in one branch of ancestral Rho1-signaling? We performed complementation tests with temperature-sensitive alleles from both ScRHO1 complementation groups (Saka et al., 2001) to answer this question. Again, we used ORF-replacements of ScRHO1 with the A. gossypii counterpart but in addition we integrated temperature-sensitive ScRHO1 alleles at the LEU2 locus. For this purpose, we used the Scrho1-2 mutant that has a defect in the actin branch of Rho1 signaling and the Scrho1-4 mutant that has a defect in the glucan biosynthesis branch of Rho1 signaling (Saka et al., 2001). We had to increase the restrictive temperature to 39°C as the published 37°C (Saka et al., 2001) did not lead to the described phenotypes in our strain background. As shown in Fig. 4D (first row), cells with the Scrho1 mutants from both complementation groups can grow at the permissive temperature of 25°C but die at the restrictive temperature of 39°C. AgRHO1a only partially complements the mutant with a defect in actin regulation (Fig. 4D, second row). Together with the Latrunculin A sensitivity of the Agrho1a deletion, this supports the hypothesis of AgRHO1a being important for actin regulation. This experiment further suggests a loss of function of AgRHO1a in glucan biosynthesis as emphasized by the failure of AgRHO1a to complement the Scrho1-4 mutant, which has a defect in glucan biosynthesis. As expected from the ability of AgRHO1b to complement the loss of ScRHO1, AgRHO1b is able to complement the defects of both Scrho1-2 and Scrho1-4 (Fig. 4D, third row).

An atypical switch I region in AgRho1a

We wanted to know whether the observed changes in AgRho1a function are reflected by changes in its protein sequence compared with AgRho1b. Therefore, we compared the sequences of the homologs with each other and with more than 300 Rho-type GTP-binding proteins from different organisms. We identified a single histidine residue at position 39 in the switch I region of AgRho1a that is a conserved tyrosine in all 323 Rho-type GTP-binding proteins we examined. This atypical switch I region is shown in an alignment with examples from Rho-proteins of other model organisms in Fig. 5A. To exclude that this atypical switch I region in AgRho1a is due only to a sequencing error or a mutation in our sample, we verified the histidine residue by resequencing the coding DNA region (not shown). We speculated that the atypical histidine might contribute to the difference in AgRHO1a and AgRHO1b function. If so, a mutant Agrho1a strain with a histidine-to-tyrosine exchange at position 39 should be able to take over the function of AgRho1b and consequently should be viable even if AgRHO1b is deleted, which is lethal in the wild-type background. To test this hypothesis, we mutated the cytosine at position 115 of Agrho1aH39Y by resequencing the coding DNA region (not shown). We speculated that the atypical histidine might contribute to the difference in AgRHO1a and AgRHO1b function. If so, a mutant Agrho1a strain with a histidine-to-tyrosine exchange at position 39 should be able to take over the function of AgRho1b and consequently should be viable even if AgRHO1b is deleted, which is lethal in the wild-type background. To test this hypothesis, we mutated the cytosine at position 115 of Agrho1a to a thymine, resulting in a change from histidine to tyrosine, analogous to the tyrosine of AgRho1b, and evaluated whether the mutated Agrho1a gains AgRho1b function. For this purpose, we integrated the mutated allele into an A. gossypii wild-type strain at the original Agrho1a locus. The resulting strain was viable (Fig. 5B) although its radial growth speed was a bit slower than wild type (Fig. 5C). We then combined this mutation with a deletion of Agrho1b. A heterokaryotic Agrho1bΔ strain was used as a control. Although spores from the strain only deleted for Agrho1b never gave rise to a mature mycelium, the presence of Agrho1aH39Y restored growth and cells formed a mature mycelium (Fig. 5B). As shown in Fig. 5C, the growth speed of this strain was slower than wild type, and the difference in growth speed was less pronounced at 37°C. We hypothesized that, unlike the wild-type Agrho1a, the mutant Agrho1a would be also capable of complementing the lethal ScRHO1 deletion. Therefore, we constructed an ORF-replacement of ScRHO1 with Agrho1aH39Y (Fig. 5D, third row).

Fig. 3. Localization of Arho1 proteins. N-terminal fusions of yellow fluorescent protein (YFP) to Arho1a and cyan fluorescent protein (CFP) to Arho1b localizing at the tip (A) and at the septum (B). The slides were incubated for 1 hour at the given temperature prior to microscopy. The overlay uses green for YFP-Arho1a and red for CFP-Arho1b. Bar, 10 μm (A); 5 μm (B). (C) Colocalization of GFP-Arho1b with 1,3-β-glucan biosynthesis at the tip. Mycelia that were grown on microscopy slides were incubated with 1 mg/ml Aniline Blue in AFM for visualization of 1,3-β-glucan biosynthesis for 5-10 minutes prior to microscopy. Aniline Blue is shown in red and GFP-Arho1b in green on the overlay image. Bar, 10 μm.
In contrast to the wild-type AgRHO1a allele (Fig. 4A,B), Agrho1aH39Y fully restores growth in a background deleted for ScRHO1, even under heat stress at 39°C (Fig. 5D). Furthermore, GFP-Agrho1aH39Y localizes in a cortical pattern that is distinct from wild-type GFP-Agrho1a and similar to CFP-Agrho1b (Fig. 5E; Fig. 3A). Together with the complementation of the growth defects of an Agrho1b and an Scrho1 deletion, this is a strong indicator for the importance of the histidine 39 of AgRho1a for divergence of protein function between the two AgRho1 proteins.

Histidine 39 of AgRho1a is responsible for GAP specificity

We wondered how a single residue could influence the function of AgRho1a in such a dramatic way. The residue that is altered in AgRho1a is located in the switch I region of the small GTP-binding protein. This is one of the regions that undergoes a nucleotide-dependent conformational change (Milburn et al., 1990). A survey of protein structures shows that the tyrosine residue, which corresponds to the atypical histidine in AgRho1a, is directly involved in interaction of RhoA, Rac1 and Cdc42 with other proteins (Dvorsky et al., 2004). Interestingly, none of the 13 different structures of protein complexes where the conserved tyrosine in the switch I region participated in the interaction interface involved an effector molecule. Instead, all complexes were formed with regulators of small GTP-binding proteins like GEFs, GDP-dissociation inhibitors (GDIs) and mainly GAPs. This suggests that the different functions of the two AgRho1-proteins are not caused by different effector interactions. Consistently, the two AgRho1-proteins do not show differences in interactions with A. gossypii homologs of known S. cerevisiae Rho1 effector proteins in a two-hybrid assay. The only difference we observed was a weak interaction between AgRho1b and AgSec3 that was not found with AgRho1a (Table 1). However, Agrho1aH39Y or Agrho1bY40H mutations did not affect this interaction. Together with the results shown in Fig. 5D, this indicates that interaction with AgSec3 does not significantly contribute to the functional differences observed for the AgRho1 proteins.

Instead, we speculated that the difference in function of the AgRho1-proteins might come from a different affinity of AgRho1a and AgRho1b to a regulatory protein, probably belonging to the class of GAPs. As two important regulators of the different branches of ScRho1 signaling in S. cerevisiae are the two GAPs ScLrg1 and ScSac7 (Lorberg et al., 2001; Schmidt et al., 2002; Watanabe et al., 2001), we set up a two-hybrid assay to test for interaction of the homologous proteins from A. gossypii with the AgRho1-proteins. We have chosen the two-hybrid system for two reasons. First, owing to the low abundance of the GAP proteins, a successful co-immunoprecipitation of the GAPs together with the AgRho1-proteins from A. gossypii cell extracts was not possible. Second, Jaitner et al. (Jaitner et al., 1997) showed for a small GTP-binding protein and its binding partner that the interaction strength measured by two-hybrid experiments correlates in a semilogarithmic fashion with the dissociation constants. As only the GTP-bound forms of the GTP-binding proteins interact with the GAP proteins, we used both the wild-type and variants that carry a glutamine-to-histidine substitution in the switch II region, which results in GTP-locked variants of the proteins (Q68H, Q69H). We preferred this allele to other GTP-locked alleles because we already had experience from two-hybrid experiments with the homologous mutant from S. cerevisiae (Schmitz et al., 2001). As shown in Fig. 6A, GFP-locked AgRho1a interacts with AgLrg1, but interaction with AgSac7 was barely detectable. This raises the question, is this interaction completely absent or is it too weak to be detected by a two-hybrid assay? We addressed these possibilities by performing a co-immunoprecipitation of the two proteins in presence of aluminium fluoride.

**Fig. 4.** Complementation of S. cerevisiae rho1 mutants by AgRHO1 genes. (A) Complementation of Scrho1Δ. The open reading frame of Scrho1Δ was replaced by either AgRHO1a or AgRHO1b in a diploid strain. After sporulation, the resulting strains were subjected to tetrad analysis. Spore growth is shown for AgRHO1a (left) and AgRHO1b (right). (B) Plasmid loss. Strains were transformed with RHO1L, a plasmid containing the URA3 marker and the Scrho1 gene with promoter and terminator, and streaked on medium without or with counterselection for URA3 by 5'-fluorotic acid. (C) Two segregants from a tetrad shown in A (right panel) were tested for temperature sensitivity at 39°C in a drop dilution assay. Cultures were grown to an OD600

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**Fig. 5.** Complementation of S. cerevisiae rho1 mutants by AgRHO1 genes. (A) Complementation of Scrho1Δ. The open reading frame of Scrho1Δ was replaced by either AgRHO1a or AgRHO1b in a diploid strain. After sporulation, the resulting strains were subjected to tetrad analysis. Spore growth is shown for AgRHO1a (left) and AgRHO1b (right). (B) Plasmid loss. Strains were transformed with RHO1L, a plasmid containing the URA3 marker and the Scrho1 gene with promoter and terminator, and streaked on medium without or with counterselection for URA3 by 5'-fluorotic acid. (C) Two segregants from a tetrad shown in A (right panel) were tested for temperature sensitivity at 39°C in a drop dilution assay. Cultures were grown to an OD600 of 1, the number of cells given below the image was spotted on agar plates and incubated for 3 days. (D) Complementation of Scrho1Δ temperature-sensitive mutants from different complementation groups by the two AgRHO1 genes. Strains carrying the wild-type Scrho1Δ allele or an allele of each Scrho1 complementation group were combined with a Scrho1 deletion (top row), Agrho1a (middle row) or Agrho1b (bottom row). A drop dilution assay of cultures grown to OD600=1 was performed. The strains were incubated at the permissive (25°C; left) or the restrictive temperature (39°C; right).
Aluminium fluoride forms a stable complex with GTP-binding proteins in the GDP-bound form and with their corresponding GAP proteins (Ahmadian et al., 1997). If there is any interaction between AgRho1a and AgSac7, the complex between GAP, GTP-binding protein and aluminium fluoride can be pulled down, even if the interaction is transient and weak. We expressed GST-tagged AgRho1a and AgRho1b and the hexahistidine-tagged GAP-domain of AgSac7 in E. coli. The GAP domain of AgSac7 can be pulled down with both AgRho1a and AgRho1b (Fig. 6B). Attempts to perform a similar experiment with AgLrg1 were unsuccessful because we were unable to obtain expression of soluble protein, even from constructs with fragments of different length and under different conditions. However, together with the two-hybrid data, the results for AgSac7 show that AgRho1a and AgSac7 do interact and can be co-purified if the complex is stabilized, but that this interaction is probably so weak that it is close to the detection limit of the two-hybrid assay.

The situation for AgRho1b is nearly the opposite to AgRho1a. GTP-AgRho1b shows only a weak interaction with AgLrg1 but a strong interaction with AgSac7 (Fig. 6A). This interaction is stronger than the background detected with a vector control, but clearly weaker than the interaction between AgSac7 and AgRho1b.

We also tested AgRho1aH39Y to see whether the mutation influences binding to a GAP protein. Indeed, the switch I mutation resulted in a stronger interaction between AgRho1a and both GAPs. Although this is obvious for AgSac7, where interaction of the protein with a wild-type switch I is barely detectable, we used 3-aminotriazole to obtain an estimate of interaction strength with AgLrg1 (Fig. 6C). In this assay, yeast cells can grow on higher concentrations of 3-aminotriazole if they produce more of the His3 reporter. Therefore, stronger interaction between bait and prey of a two-hybrid assay leads to higher resistance against 3-aminotriazole. Although yeast cells with the AgRho1a wild-type protein as a bait and AgLrg1 as a prey do not grow on 2 mM 3-aminotriazole, a strain with AgRho1aH39Y can grow even on 5 mM 3-aminotriazole, indicating a much stronger interaction. This change in GAP-interaction strength is probably the reason for the capability of an AgRho1aH39Y mutant to take over AgRho1b function.

Table 1. Interaction of AgRho1 proteins with potential effector proteins

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The situation for AgRho1b is nearly the opposite to AgRho1a. GTP-AgRho1b shows only a weak interaction with AgLrg1 but a strong interaction with AgSac7 (Fig. 6A). This interaction is stronger than the background detected with a vector control, but clearly weaker than the interaction between AgSac7 and AgRho1b.

We also tested AgRho1aH39Y to see whether the mutation influences binding to a GAP protein. Indeed, the switch I mutation resulted in a stronger interaction between AgRho1a and both GAPs. Although this is obvious for AgSac7, where interaction of the protein with a wild-type switch I is barely detectable, we used 3-aminotriazole to obtain an estimate of interaction strength with AgLrg1 (Fig. 6C). In this assay, yeast cells can grow on higher concentrations of 3-aminotriazole if they produce more of the His3 reporter. Therefore, stronger interaction between bait and prey of a two-hybrid assay leads to higher resistance against 3-aminotriazole. Although yeast cells with the AgRho1a wild-type protein as a bait and AgLrg1 as a prey do not grow on 2 mM 3-aminotriazole, a strain with AgRho1aH39Y can grow even on 5 mM 3-aminotriazole, indicating a much stronger interaction. This change in GAP-interaction strength is probably the reason for the capability of an AgRho1aH39Y mutant to take over AgRho1b function.
As the position 39 (taking AgRho1a numbers as a reference) is not the only residue that is different between the AgRho1 proteins, we asked whether there are other residues involved in the different GAP interactions. We looked at the 27 residues that are known to form the interaction interface with GAP proteins from structural studies of homologous proteins (Dvorsky et al., 2004). In addition to position 39, only three of these (positions 45, 98 and 139) differ between AgRho1a and AgRho1b. Only one of these (position 45) is located in the switch I region (Fig. 5A). Interestingly, the glutamic acid at that position in AgRho1a is also found in S. cerevisiae Rho1, whereas the aspartic acid of AgRho1b is conserved in Rho1 of Kluyveromyces lactis. Therefore, we tested these proteins for interaction with the A. gossypii GAPs (Fig. 6D). Although there is hardly any interaction with both GAPs and K/Rho1, ScRho1 interacts with AgLrg1 but not with AgSac7. In addition, we mutated tyrosine 39 of ScRho1 to histidine to mimic the switch I region of AgRho1a (Y39H). In agreement with our findings from above the Y39H mutation weakens the interaction with AgLrg1. These results suggest that in addition to the residue at position 39, other residues might contribute to the difference in GAP interaction of AgRho1a and AgRho1b. However, this may not be the residue at position 45 because this residue is identical in AgRho1b and K/Rho1 but the latter does not interact with any of the A. gossypii GAP-proteins. Instead it might be that the interaction surface of the AgRho1 proteins and their GAPs might include different residues, which also requires adaptations in the sequence of the GAPs in addition to differences in the sequence of the Rho proteins. To determine whether there is a different regulation of the two AgRho1 proteins by the GAPs, as suggested by the interaction studies, we constructed strains carrying a mutation that encodes dominant active forms of AgRho1a and AgRho1b. We then compared the phenotypes of these strains with phenotypes of strains deleted for either AgSac7 or AgLrg1. Removal of a GAP from the regulatory circuit of a small GTP-binding protein leads to an accumulation of a GTP-bound Rho protein and therefore to a phenotype that is similar to the GTPase-deficient form of the small GTP-binding protein. A deletion of AgLrg1 or a GAP-deficient allele of AgRho1a resulted in slow growth compared with wild type. However, deletion of AgSac7 or introduction of a GAP-deficient allele of AgRHO1b was lethal (Fig. 7A). Together with the interaction data, these phenotypes support the idea that AgRho1a is the preferred target of AgLrg1, whereas AgRho1b might be the preferred target of AgSac7.

Our results suggest that GAP-mediated GTP hydrolysis of AgRho1a should be lower than GTP hydrolysis of AgRho1b in presence of AgSac7. To test this, we expressed the GAP-binding proteins and the GAP domains of these A. gossypii proteins in E. coli and purified them for an in vitro GAP assay (Fig. 7B). The assay we used detects the release of phosphate from GTP hydrolysis by a color reaction that can be monitored by an increase in absorbance at a wavelength of 650 nm. GTP hydrolysis of all control reactions, each GAP-binding protein and the GAP on its own was below OD 0.1. We used the GAP-GTP-binding protein pair human p50RhoGAP and RhoA that was supplied with the assay as a positive control. RhoA had the highest rate of GTP hydrolysis with both human p50RhoGAP and the AgSac7 GAP domain. With both of these GAP proteins, GTP hydrolysis of AgRho1b was significantly higher than GTP hydrolysis of AgRho1a. The interaction data, together with genetics and the GAP assay, suggest that both GAPs still are able to act on both GAP-binding proteins. However, this raises the question, is the GAP with the higher or the GAP with the lower affinity the regulator of a Rho protein in vivo? Both situations seem to be possible. A higher affinity would result in a greater specificity of the reaction, whereas a lower affinity would result in an increase of the GTP-bound form of the Rho protein. An answer to this question might come from the localization of the GAP.

**Fig. 6.** Rho proteins and GAP interactions. (A) Two-hybrid analysis of the AgRho1 proteins and potential GAP-activating proteins. The C-terminal lipid-modification motif of the small GTP-binding proteins was truncated from the coding sequence to allow two-hybrid testing. The glutamate-to-histidine exchange in switch II mimics the GTP-bound state of the proteins. The histidine-to-tyrosine exchange is indicated by H39Y, the reverse mutation in AgRho1b by Y40H. The left panel shows a growth control on medium selective for prey and bait plasmids. The medium in the right panel is also selective for interaction. (B) Pull-down experiment in the presence of aluminium fluoride. Glutathion-S-Transferase (GST) or AgRho1a proteins fused to GST were bound to glutathion-sepharose and washed. The eluted proteins were subjected to western blot analysis. The blot was probed with α-GST and α-HIS. (C) The H39Y mutation increases interaction strength of the indicated prey/bait combinations from A were additionally tested on medium with different concentrations of 3-amino triazole to visualize the increase in interaction strength of the AgRho1aH39Y mutant. The first image on the left side is the growth control. (D) Two-hybrid analysis of Rho1 proteins from S. cerevisiae and Kluyveromyces lactis. The procedure used was identical to the one described for A.
proteins. To investigate this, we fused GFP to *AgLrg1* and *AgSac7*. *AgLrg1*-GFP localized to the cortex of the hyphal tip, resembling the localization pattern of *AgRho1b*. *AgSac7*-GFP was found in the cytoplasm similar to *AgRho1a* (Fig. 7C). This favors the second situation described above. *AgLrg1* is probably part of the regulatory circuit of *AgRho1b*. The purpose of its high affinity towards *AgRho1a* might be to inactivate *AgRho1a* efficiently at the tip region. The same may be true for *AgSac7* and *AgRho1b*. *AgSac7* probably regulates *AgRho1a* and might prevent activity of *AgRho1b* when *AgRho1b* is not at the tip.

In summary, our results show that *AgRho1a* function has evolved by mutation of a single residue in the switch I region, leading to a situation where *AgLrg1* regulates *AgRho1a* and *AgSac7* regulates *AgRho1b*, which in turn results in divergence of protein localization and function.

**Discussion**

The initial question we addressed in this study was how the function of a Rho-type GTP-binding protein diverges after gene duplication. We found that during evolution, alteration of a single tyrosine to a histidine in the switch I region of one of the two Rho1 proteins encoded by the filamentous fungus *A. gossypii* can influence interaction with GTPase-activating proteins. This has an effect on protein function without altering the specificity for effector proteins. The finding that GTP-binding protein function evolves by mutation of the switch region is remarkable because this region, and especially the tyrosine at position 39 (*AgRho1b* numbering), is highly conserved. By contrast the large variety of Rho-GTP binding proteins has probably evolved by mutation of the more variable parts of the interaction interface (Wherlock and Mellor, 2002). There are only a few reports about small GTP-binding proteins where this is not the case: Heo and Meyer (Heo and Meyer, 2003) developed an algorithm to generate mutations that lead to a switch of Rho-protein function. They found that mutations outside the effector interaction interface can induce a switch of function. However, these switch of function mutations were also outside the conserved switch regions of the GTP-binding proteins. The latter is also true for a group of three closely related Rac proteins that have different functions (Haesauser et al., 2003). In this study, the residues that are thought to be responsible for the different functionality have an effect on the nucleotide binding capability and a different activation by a GEF protein but they are also located outside the switch region.

What is the effect of the differences in *AgRho1a* and *AgRho1b* sequence? We found that especially the histidine at position 39 in the switch I region of *AgRho1a* represents a mutation that occurred during evolution and alters interaction of *AgRho1a* with regulatory proteins, namely with GAPs. The homologous residue in the 323 other Rho-proteins we checked (including *AgRho1b* and *ScRho1*) was without exception a conserved tyrosine. Even though there are other differences between *AgRho1a* and *AgRho1b*, it was possible to revert *AgRho1a* function to *AgRho1b* function by simply changing histidine 39 of *AgRho1a* to a tyrosine. The resulting *AgRho1aH39Y* was able to compensate for the loss of *AgRho1b*, whereas an *AgRho1b* deletion alone was lethal (Fig. 5B, C). The localization pattern of *AgRho1aH39Y* mimicked that of *AgRho1b* (Fig. 5E). The atypical histidine 39 of *AgRho1a* is found in the switch I region, which is known to interact with GAPs in various small GTP-binding proteins (Dvorsky et al., 2004). For this reason, we investigated the interactions of *AgRho1a* and *AgRho1b* with the two Rho1-GAPs *AgLrg1* and *AgSac7*. We found that Rho1a interacts preferentially with *AgLrg1* and weakly with *AgSac7*.
whereas AgRho1b shows strong interaction with both GAPs (Fig. 6). The difference in two-hybrid interaction strength is reflected by the ability of AgSac7 to activate GTP-hydrolysis more efficiently for AgRho1b than for AgRho1a as shown in an in vitro GTPase activity assay (Fig. 7). Interestingly, this strong interaction with the Rho GAPs seems to be crucial for AgRho1b function, as AgRho1aH39Y can provide cell viability in absence of AgRho1b and shows GAP interaction similar to AgRho1b. This finding implies that precisely controlled GTP-hydrolysis is crucial for the different AgRho1 functions. Small GTP-binding proteins do not only work as on/off switches. It has been shown that the cycling between the GDP- and the GTP-bound form is important for their functionality (Gladfelter et al., 2002). Evidence for this theory comes from findings that cycling of ScCdc42 is important for its function (Gladfelter et al., 2002). Interestingly, an Sccdc42 mutant that was derived from an in vitro mutagenesis has an exchange of tyrosine for histidine in the switch I region like it is present in AgRho1a (Gladfelter et al., 2002). In agreement with our results, this Sccdc42 mutant could not be activated to the same level of GTPase activity by its GAP compared with wild-type ScCdc42 with a tyrosine in switch I. Therefore, AgRho1aH39Y might be able to compensate for AgRho1b loss by having similar GDP/GTP cycling properties as AgRho1b and thus mimic AgRho1b action. Such a scenario would suggest that if both AgRho1 proteins are present at the same time together with one of the GAPs, the Rho protein with the lower affinity towards the GAP would be active at a location where it usually is completely inactive. Therefore, this phenotype correlates with the Aghro1bQ69H mutant. The same would be the case for the Aglrg1a strain and Aghrho1aQ68H. This would also mean that the respective roles of the two GAPs are conserved between A. gossypii and S. cerevisiae, because ScLrg1 primarily regulates biosynthesis of 1,3-β-glucan and ScSac7 primarily regulates the Pkc1 branch of Rho1 signaling (Watanabe et al., 2001). Such a hypothesis is supported by the data we obtained from deletion analysis, localization and complementation studies (Figs 1-4). All these indicate that AgRho1a is involved in actin regulation but has lost the glucan biosynthesis function that is preserved in ScRho1 and AgRho1b, as shown by complementation of S. cerevisiae mutants (Fig. 4). In addition AgRho1a gained a novel function that prevents cells from lysis at low temperature.

The fact that both Rho1 proteins have different functions in A. gossypii raises the question, why could this be an advantage for the cell? Different aspects have to be taken into account when answering this question. First, there are more Rho1 regulators in S. cerevisiae than in A. gossypii. Fig. 8 compares the core machinery of the Rho1 regulatory network of both organisms. The GEFs ScRom1 and ScRom2 (Ozaki et al., 1996), as well as the GAPs ScSac7 and ScBag7 (Schmidt et al., 2002), are so-called ‘Twin-ORFs’, remnants of a whole genome duplication that occurred in the S. cerevisiae lineage (Dietrich et al., 2004). These proteins add a level of complexity to ScRho1 regulation and thus might allow the adaptation of activity of ScRho1 to a variety of intra- and extracellular signals. This observation leads to a simple explanation for the AgRHO1 duplication: it might have been maintained in the genomes of various species because it simply expands the organisms’ capacity to fine-tune Rho1 activity to a similar level as with duplication of several GAP or GEF proteins. In good agreement with such a hypothesis is the fact that a tandem RHO1 duplication is also found in Kluveromyces waltii, Saccharomyces kluyveri and Holleya sinecauda (Cliften et al., 2003; Kellis et al., 2004; Walther and Wendland, 2005). Like A. gossypii, K. waltii and S. kluyveri have not undergone the whole genome duplication that is found with S. cerevisiae, and therefore also have fewer GAP and GEF proteins. Even though there is no genome sequence available for H. sinecauda, the close evolutionary relationship to A. gossypii suggests a non-duplicated genome.

Another reason why it might be advantageous for A. gossypii to have two Rho1 proteins might be due to the filamentous growth of A. gossypii. In S. cerevisiae, cell cycle and cell division are tightly coupled (Philipppsen et al., 2005). The major part of cell wall expansion and thus 1,3-β-glucan biosynthesis occurs during bud growth. The duration of this phase is regulated by the cell-cycle machinery, which means that full Rho1 activity in 1,3-β-glucan biosynthesis is required only for a specific period during the cell cycle. Therefore, the different branches of ScRho1 activity might be temporally restricted. This temporal restriction of polar growth is not present in A. gossypii, rather polar growth is active in the filamentous fungus all the time (Philipppsen et al., 2005). The mycelium even maintains many growing tips at the same time and tip growth is independent from the asynchronous nuclear cycles (Gladfelter et al., 2006). This tremendous polarized growth requires
Materials and Methods

Nomenclature
According to the guidelines used for the A. gossypii gene annotation, we refer to ABR185W as AgRho1a and to ABR182W as AgRho1b, whereas in an initial characterization the authors referred to ABR185W as RHO1 and ABR182W as RHO1 (Wendland and Philippsen, 2001; Walther and Wendland, 2005). However, these species have another small GTP-binding protein involved in control of the actin cytoskeleton (Borkovich et al., 2004); a Rac1 homolog that is missing in all species closely related to S. cerevisiae and therefore also in A. gossypii. This suggests that some filamentous fungi might use different strategies to achieve fast filamentous growth.

In summary, we show here that a simple mutation in the switch I region of a GTP-binding protein can change its affinity towards its GTPs, which probably lead to slight variations of the GDP-GTP cycling and finally leads to a decoupling of still very similar protein function without impairing effector interaction.

Ashbya gossypii strains and growth conditions
All strains are listed in supplementary material Table S1. Deletion strains were constructed by PCR-based gene targeting, according to the method described by Wendland et al. (Wendland et al., 2000). All oligonucleotides are listed in supplementary material Table S3. Strains were grown at the indicated temperatures either in AFM (Ashbya Full Medium) with or without Geneticin or CloNAT, or in synthetic medium when selecting for auxotrophic markers (Knechtle et al., 2003).

Saccharomyces cerevisiae strains and growth conditions
The genotypes of all strains used in this study are listed in supplementary material Table S2. S. cerevisiae strains were grown at 30°C if not indicated otherwise. Media were used according to Amberg et al. (Amberg et al., 2005).

DNA manipulations and sequencing
All DNA manipulations were carried out according to Sambrook et al. (Sambrook and Russell, 2002). Purification of the hexahistidine-tagged AgSac7p was performed using an AKTA FPLC system (GE Healthcare Life Sciences, Pittsburgh, USA) with 1 ml HisTrap FF column according to the instructions of the manufacturer. A Coomassie stained SDS-page of the purified proteins is shown in supplementary material Fig. S1.

Pulldown assays
GST pulldown of GAP and GTP-binding proteins was performed in the presence of aluminium fluoride by addition of 10 mM NaF and 450 μM AlCl3 to lysis and washing buffers for stabilization of the complex. Apart from the addition of aluminium fluoride, buffers and conditions used were similar to the purification of GST-tagged proteins described above. Western blots were probed using anti-GST from rabbit (G7781 Sigma, St Louis, USA) and anti-Penta-HIS from mouse (34660 Qiagen, Hilden, Germany) as primary and anti-rabbit coupled to IRdye800 (926-32210, Li-Cor, Lincoln, USA) as secondary antibodies. Signals were detected with an Odyssey infrared imaging system (Li-Cor, Lincoln, USA).

Western blotting for detection of AgSac7-GFP
Western blots were probed with a 1:1000 dilution of antibodies directed against GFP (11 814 460 001 Roche, Mannheim, Germany).

In vitro GAP assay
The in vitro GAP assay was performed using the RhoGAP assay Biochem Kit (BK105, Cytoskeleton, Denver, CO). Rho A and p50RhoGAP were supplied with the kit.

References


