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Interfacial Self-Assembly of a Fungal Hydrophobin into a Hydrophobic Rodlet Layer

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The Sc3p hydrophobin of the basidiomycete *Schizophyllum commune* is a small hydrophobic protein (100 to 101 amino acids) containing eight cysteine residues. Large amounts of the protein are excreted into the culture medium as monomers, but in the walls of aerial hyphae, the protein is present as an SDS-insoluble complex. In this study, we show that the Sc3p hydrophobin spontaneously assembles into an SDS-insoluble protein membrane on the surface of gas bubbles or when dried down on a hydrophilic surface. Electron microscopy of the assembled hydrophobin shows a surface consisting of rodlets spaced 10 nm apart, which is similar to those rodlets seen on the surface of aerial hyphae. When the purified Sc3p hydrophobin assembles on a hydrophilic surface, a surface is exposed with high hydrophobicity, similar to that of aerial hyphae. The rodlet layer, assembled in vivo and in vitro, can be disassembled by dissolution in trifluoroacetic acid and, after removal of the acid, reassembled into a rodlet layer. We propose, therefore, that the hydrophobic rodlet layer on aerial hyphae arises by interfacial self-assembly of Sc3p hydrophobin monomers, involving noncovalent interactions only. Submerged hyphae merely excrete monomers because these hyphae are not exposed to a water-air interface. The generally observed rodlet layers on fungal spores may arise in a similar way.

INTRODUCTION

Filamentous fungi grow by means of hyphae that extend at their apices and ramify into a mycelium that penetrates moist substrates. After a period of assimilative growth, hyphae may grow out of the substrate to form aerial hyphae and various reproductive structures. In the basidiomycete *Schizophyllum commune*, the formation of aerial hyphae is accompanied by expression of the *Sc3* gene in the mycelium, with its mRNA accumulating to ~1% of the total RNA mass (Mulder and Wessels, 1986). The activity of this gene remains very low in a mutant (*thin*) that forms few, if any, aerial hyphae (Wessels et al., 1991a). The *Sc3* gene belongs to a family of genes in Schizophyllum that encodes small hydrophobic proteins, which are characterized by the presence of eight cysteine residues at conserved positions, common hydropathy patterns, and signal sequences for secretion (Schuren and Wessels, 1990). We have coined the name hydrophobins for these proteins, although the term was used earlier to denote any hydrophobic substance covering microbial cells (Rosenberg and Kjelleberg, 1986).

At the time of formation of aerial hyphae, the Sc3p hydrophobin (100 to 101 amino acids, of which 39 are nonpolar) is excreted into the medium (Wessels et al., 1991a, 1991b). However, in aerial hyphae the hydrophobin is found in the cell walls as a highly insoluble complex that can only be dissociated by agents such as performic acid (Wessels et al., 1991a) or trifluoroacetic acid (TFA) (de Vries et al., 1993). Two other hydrophobin genes, *Sc1* and *Sc4*, are active only in dikaryons of Schizophyllum. The hydrophobin encoded by *Sc4* has been identified in the medium and, as an insoluble aggregate, in the walls of hyphae that make up the context of fruit bodies (Wessels et al., 1991b).

In this study, we report that Sc3p hydrophobin monomers spontaneously assemble at a water–air interface into an insoluble protein layer with the appearance of typical hydrophobic rodlet structures similar to those seen on surfaces of aerial hyphae of Schizophyllum and many hydrophobic fungal spores. We propose a model for the appearance of this hydrophobic rodlet layer on walls of air-exposed fungal structures.

RESULTS

Sc3p Forms SDS-Insoluble Complexes when Exposed to a Water-Gas Interface

In a 5-day-old standing culture of the wild-type monokaryon of Schizophyllum, labeled with ^{35}S-sulfate, Sc3p appeared to be the main, labeled protein in the medium, as shown in Figure 1 (lane 1). After oxidizing with performic acid and converting cysteine residues into cysteic acid residues, the apparent molecular mass of the protein on these gels shifted from 24 to 28 kD (de Vries et al., 1993). This oxidized protein was originally used for partial N-terminal amino acid sequencing to

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identify it as the product of the *Sc3* gene (Wessels et al., 1991a, 1991b). To investigate the possibility that the insoluble form of Sc3p in the walls of aerial hyphae arose by formation of disulfide bridges between the monomers seen at the 24-kD position, the medium was subjected to various oxidative treatments. Among these was oxygenation by bubbling oxygen or air through the medium for 4 hr; bubbling with nitrogen gas served as a control. It was found that, irrespective of the gas used, Sc3p was converted into an SDS-insoluble product that did not enter the separation gel, with most of the radioactive material remaining on top of the stacking gel (Figure 1, lane 2). The presence of 100 mM DTT during gas bubbling did not influence the results.

Heating the medium to 100°C and holding it at this temperature for 10 min also effected aggregation of Sc3p. SDS-PAGE analysis showed aggregates that remained at the top of the stacking gel (Figure 1, lane 3). Aggregation of Sc3p was not observed during 30-min incubations at temperatures below 80°C. Whether aggregation was caused by the high temperature or by small gas bubbles escaping from the medium is not yet clear.

Freezing the medium in liquid nitrogen or at −20°C also resulted in aggregation of Sc3p, although this was less efficient than heating (data not shown). However, a 100-fold concentration of the medium at room temperature by ultrafiltration was ineffective in causing aggregation.

In shaken cultures (225 rpm), air is constantly swirled into the culture medium. In agreement with Sc3p aggregating at a water–air interface, little monomeric Sc3p was found in the medium of such cultures. After the addition of a small quantity of radioactive Sc3p monomers, the labeled Sc3p aggregated during shaking but did not become associated with the hyphae; the medium with aggregated Sc3p could be filtered through filter paper after which the aggregated Sc3p could be recovered from the filtrate by centrifugation.

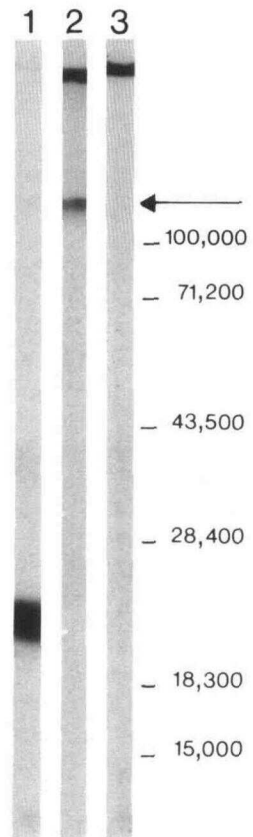

Figure 1. SDS-PAGE Autoradiograph of ^{35}S-Labeled Medium Proteins.

Lane 1 contains medium proteins isolated from a standing culture; lane 2, medium proteins after bubbling gases through the medium; lane 3, medium proteins after heating the medium. Molecular weight markers are given at right. Arrow indicates the border between stacking and separation gel.

Solubility of Aggregated Sc3p in Solvents

Similar to Sc3p in the walls of aerial hyphae (Wessels et al. 1991a; de Vries et al. 1993), aggregated Sc3p from the culture medium was insoluble in hot 2% SDS, 8 M urea, and organic solvents (ethanol, methanol, chloroform/methanol [3:1], and acetone) but could be solubilized and dissociated with performic acid or TFA. After evaporation of TFA, TFA-treated Sc3p was soluble in water, in 0.1 M Tris-HCl, pH 8.0, in SDS (2%) buffered with 0.1 M Tris, pH 8.0, in 0 to 60% ethanol, in 0 to 50% acetone, and in 0 to 60% acetonitrile.

When solutions of TFA-treated Sc3p in 40% acetone or 50% acetonitrile were shaken overnight at 225 rpm at room temperature, 37% and 59%, respectively, of the Sc3p aggregated. However, shaking in 50% ethanol caused no aggregation of Sc3p. On the other hand, TFA-treated Sc3p in aqueous solutions (without SDS) aggregated almost instantaneously upon shaking.

Purification of Sc3p from the Medium

The determined solubility characteristics of Sc3p were used for purification of the protein (see Methods). Centrifugation of the filtered medium from standing cultures of the wild-type monokaryon of Schizophyllum resulted in a small pellet that contained some aggregated Sc3p. Vigorous agitation of the clear supernatant in a blender resulted in the formation of turbidity due to aggregates of Sc3p that could then be recovered by centrifugation. SDS-PAGE of the TFA-treated precipitate and silver staining of the gels showed intense bands at 25 kD (Sc3p) and 15 kD, as shown in Figure 2 (lane 1; note that in the Phast-system used here, Sc3p has a somewhat lower mobility than in the conventional PAGE shown in Figure 1). Some additional material (~5% by weight) showing the same electrophoretic pattern could be obtained by subsequently heating the

medium, but this material was discarded. The precipitated 15-kD protein was not labeled when ^{35}S-sulfate was included in the medium, which explains why only Sc3p was observed after labeling experiments (Figure 1, lane 1). After heating, the final supernatant contained a variety of proteins in small quantities (results not shown).

For further purification, the air-aggregated material was taken up in TFA and insolubles were removed by centrifugation. After evaporation of TFA, the residue was taken up in 60% ethanol, resulting in the selective solubilization of Sc3p (Figure 2, lanes 2 and 3). After removal of the 15-kD protein by centrifugation, the ethanol in the supernatant was removed by dialysis. Pure Sc3p could then be aggregated by heating or bubbling the aqueous solution.

Formation of Air Vesicles by Sc3p

When bubbling an aqueous solution of purified Sc3p with nitrogen gas, a suspension was obtained that had a milky appearance and showed irregularly shaped gas vesicles in the light microscope, as shown in Figure 3A. By applying a vacuum, these structures collapsed, resulting in a clear solution with aggregated Sc3p present on the water surface and sticking to the glass surface. The aggregated Sc3p was insoluble in hot SDS sample buffer. Air-aggregated material from a culture medium always contained some lipid material, which was removed by extraction with chloroform/methanol. When Sc3p purified from lipid-extracted, air-aggregated material was used, the same results were obtained. Sc3p purified from TFA-treated lipid-extracted walls of aerial hyphae also coated gas bubbles with an SDS-insoluble aggregate.

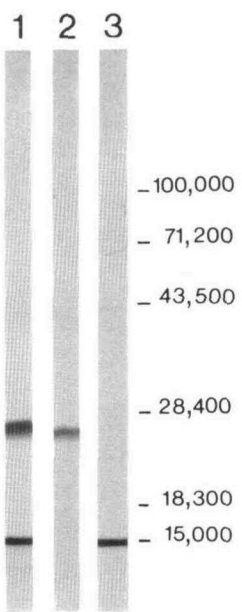

Figure 2. SDS-PAGE of Fractions during Purification of Sc3p.

Following SDS-PAGE, the gels were silver stained. Lane 1 contains proteins that were aggregated by frothing the culture medium and then dissolving in TFA; lane 2, protein subsequently soluble in 60% ethanol; lane 3, protein insoluble in 60% ethanol. Molecular weight markers are given at the right.

Electron Microscopy of Aggregated Sc3p Shows Membranes with a Rodlet-Decorated Surface

When air vesicles coated with aggregated Sc3p were freeze fractured (Figure 3B) or directly shadowed after centrifugation, which was accompanied by disruption of the vesicles (Figure 3C), a mosaic pattern of parallel rodlets was seen with a rodlet spacing of ~10 nm. Similar results were obtained when monomers of Sc3p were heated (results not shown). Rodlets were also seen when an aqueous solution of Sc3p monomers was dried down on a Formvar-coated grid followed by shadowing (Figure 3D). The same results were obtained by using lipid-extracted Sc3p preparations (data not shown).

Negatively stained preparations of assembled, purified Sc3p showed short fibers with a diameter of 10 nm (Figure 3E), similar in dimensions to the rodlets seen in shadowed surfaces.

When purified Sc3p from walls of aerial hyphae, instead of from the medium, was used to examine the morphological appearance of assembled Sc3p, the results were the same as those obtained with the medium proteins (results not shown). This suggests that Sc3p molecules obtained from both sources are identical. Similar rodlets were formed when the medium of 5-day-old cultures of Schizophyllum were shaken, i.e., without prior purification of Sc3p.

Thin sections of assembled, purified Sc3p, which were pelleted by centrifugation, showed long 10-nm-thick profiles (Figure 3F). Serial sections indicated that these profiles represent sections through membranes formed by assembled Sc3p.

Rodlets Similar to Those Generated by Sc3p Are Present on the Surface of Aerial Hyphae

Rodlets similar to those generated by purified Sc3p were observed earlier on the surface of hyphal wall preparations of Schizophyllum (Wessels et al., 1972). However, no distinction was made between walls derived from aerial or from submerged hyphae. Therefore, we reexamined the freeze-fractured and freeze-etched surfaces of isolated aerial and submerged hyphae of the wild-type monokaryon of Schizophyllum and of hyphae of the *thin* mutant, which does not form aerial hyphae (Wessels et al., 1991a). It has been shown previously that only aerial hyphae contain SDS-insoluble Sc3p (Wessels et al. 1991a, 1991b). We also examined basidiospores of Schizophyllum, which after boiling with 2% SDS failed to show the

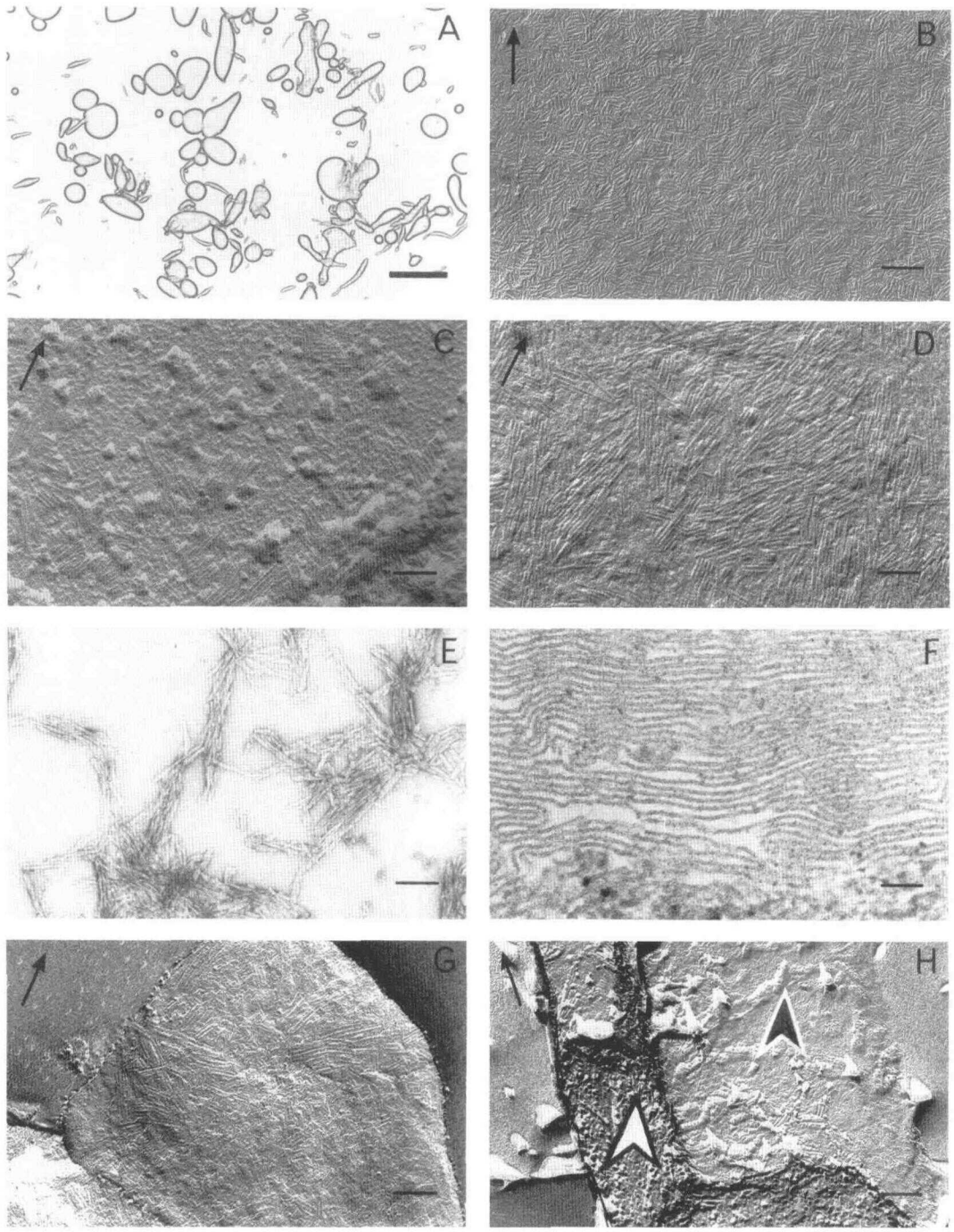

Figure 3. Morphological Appearance of Assembled Sc3p.

(A) Light microscopy of gas vesicles coated with self-assembled, purified Sc3p.
(B) Freeze fracture of Sc3p-coated gas vesicles.
(C) Shadowing of pelleted assemblages of purified Sc3p after bubbling.
(D) Direct shadowing of an aqueous solution of purified Sc3p dried down on a Formvar-coated grid.
(E) Negatively stained preparation of assembled, purified Sc3p.
(F) Thin section of pelleted, assembled, and purified Sc3p.
(G) Freeze-fractured surface of an aerial hypha.
(H) As shown in **(G)** but freeze etched. The white arrowhead indicates the fracture through the cell wall; the black arrowhead indicates the etched surface. Thin bars in **(B)** to **(H)** = 50 μm; thick bar in **(A)** = 100 nm. Arrows at top left corners of photographs indicate the direction of shadowing.

presence of Sc3p in TFA extracts (results not shown). Rodlets were not found on walls of submerged hyphae or on hyphae of the *thin* mutant and on basidiospores. Rodlets were only observed on walls of aerial hyphae of the wild-type strain both on the fractured (Figure 3G) and the etched surface (Figure 3H). On the etched surface, rodlets were often seen in patches, probably obscured at other places by other covering material. We assume that the fracture plane often follows the hydrophobic surface and only then is an uninterrupted rodlet layer exposed (Figure 3G).

Assembled Sc3p Provides the Surface of Aerial Hyphae with a Hydrophobic Layer

Aerial hyphae are typically hydrophobic. Contact angles of 1-μL water droplets (van der Mei et al., 1991) placed on the surface of the wild-type monokaryon of Schizophyllum were 115° ± 10°, whereas those placed on the surface of the *thin* mutant without aerial hyphae were 40° ± 5°. Purified Sc3p that was aggregated on a hydrophilic glass surface produced a surface with a hydrophobicity corresponding to contact angles up to 95°. When a solution of purified Sc3p was dried down on the surface of a piece of *thin* mycelium, the hydrophobicity of this surface rose to 110° ± 10°. An exhaustive lipid extraction (chloroform/methanol, [2:1]), which does not remove the Sc3p hydrophobin, did not detectably change the hydrophobicity of aerial hyphae. It thus appears that the presence of the self-assembled Sc3p can largely explain the hydrophobicity of aerial hyphae.

DISCUSSION

Because the *Sc3* gene of Schizophyllum is activated at the time of formation of aerial hyphae (Mulder and Wessels, 1986) and because the *thin* mutation, which suppresses activation of *Sc3*, has a phenotype with few aerial hyphae, an essential role for the encoded hydrophobin in formation of aerial hyphae was envisaged (Wessels et al., 1991a). This report deals with the relationship between the hydrophobin present in the culture medium as a monomer and that present as an SDS-insoluble complex in cell walls of aerial hyphae.

Because the Sc3p hydrophobin could be solubilized from the walls by a brief treatment with formic acid in the cold but dissociated into monomers only after oxidizing all eight cysteine residues in the protein (Schuren and Wessels, 1990) to cysteic acid with performic acid (Wessels et al., 1991a, 1991b), we originally considered the presence of intermolecular disulfide bridges to be important in the formation of the insoluble hydrophobin complex. However, both dissolution and dissociation into monomers could also be achieved by a brief treatment with TFA in the cold, and no free sulfhydryl groups could be detected in monomers isolated from the medium or from the wall (de Vries et al., 1993). This indicated that noncovalent interactions between the monomers were responsible for formation of the insoluble complex. As far as we can tell at the present time, Sc3p in the medium and in the insoluble wall complex are identical.

As shown in this study, the purified Sc3p derived from the medium or from isolated walls spontaneously assembles into an SDS-insoluble complex by the presence of a water–gas interface such as that created by bubbling gases through an aqueous solution of the monomers. Sc3p then forms a protein membrane around the gas bubbles, creating gas vesicles of variable shapes, probably by lowering the surface tension. Aggregation on a water–air interface has also been used for the isolation of cerato-ulmin, the presumed wilting toxin from *Ceratocystis ulmi* that causes Dutch elm disease (Takai and Richards, 1978; Russo et al., 1982), and cryparin, a possible wilting toxin from *Cryphonectria parasitica* that causes chestnut blight (Carpenter et al., 1992). Interestingly, the amino acid sequence of cerato-ulmin determined by M. Yaguchi and coworkers (see in Bolyard and Sticklen, 1992) shows similarities to Sc3p and other hydrophobins (Stringer and Timberlake, 1993), whereas the cryparin sequence is quite similar to that of cerato-ulmin (Zhang et al., 1993).

Although clearly related to hydrophobins, particularly with respect to the spacing of the eight cysteine residues, the assemblages formed by these proteins are less stable than those formed by Sc3p and dissociate readily in water and aqueous ethanol. The Sc3p assemblage can only be dissociated by agents such as TFA, after which the monomers are soluble in water, aqueous ethanol, and detergents. After dissolution of the monomers in water, the process of interfacial assembly can be repeated, but it is prevented by the presence of lipid solvents or detergents. We surmise that during interfacial assemblage of Sc3p, a very stable conformational change occurs, such that in the formed membrane, polar groups of the protein subunits are oriented toward the water phase, whereas apolar groups face the hydrophobic gas phase.

When the assembled Sc3p hydrophobin was examined by electron microscopy, its surface appeared to be composed of parallel rodlets spaced ~10 nm apart in a typical mosaic pattern. If the protein sheets are indeed polarized, then we suspect that the rodlet surface represents the hydrophobic phase because the plane of freeze fracturing generally follows this surface. In addition, direct shadowing of Sc3p preparations dried down on a Formvar-coated grid shows the rodlet surface oriented toward the air.

Significantly, after freeze fracturing and freeze etching, similar rodlets were seen on the surface of aerial hyphae, which are known to contain the insoluble form of the Sc3p hydrophobin (Wessels et al., 1991a). Such rodlets were absent on the surfaces of submerged hyphae of the wild-type strain, hyphae of the *thin* mutant, and basidiospores: all of which are devoid of Sc3p. The assembled Sc3p is probably solely responsible for the high hydrophobicity of aerial hyphae, as shown by assembling the purified Sc3p on a glass surface; the Sc3p-coated surface became nearly as hydrophobic as the surface of aerial hyphae. Moreover, hyphae of the *thin* mutant on which a solution of Sc3p monomers was dried down attained the same high hydrophobicity as wild-type aerial hyphae. We

propose, therefore, that the rodlet layer seen at the surface of aerial hyphae confers hydrophobicity to the surface of these hyphae and that this layer arises by self-assembly of Sc3p monomers at the hydrophilic–hydrophobic, or wall–air, interface.

Rodlets of the type described here were first seen on spores of various fungi (Sassen et al., 1967; Hess and Stocks, 1969; Bronchart and Demoulin, 1971; Cole, 1971, 1973; Ghiorse and Edwards, 1973; Hashimoto et al., 1976; Beever and Dempsey, 1978; Cole and Samson, 1979). They were found to consist of protein (Hashimoto et al., 1976; Beever, 1979). Beever and Dempsey (1978) observed that a mutant of *Neurospora crassa* (easily wettable, *eas*), which produced nonhydrophobic conidiospores, lacked the spore rodlet layer. Recently, Lauter et al. (1992) identified in *N. crassa* a gene activated during light-induced conidiation, while Bell-Pedersen et al. (1992) identified the same gene as rhythmically induced during circadian zonation of colonies. This gene encodes a protein clearly homologous to the hydrophobins of Schizophyllum (Schuren and Wessels, 1990), and both groups showed that disruption of this gene caused the *eas* phenotype, whereas the wild-type gene could complement the *eas* mutation. Similarly, Stringer et al. (1991) obtained a mutant of *Aspergillus nidulans* with nonhydrophobic spores lacking rodlets through targeted inactivation of a gene with clear homology to the Schizophyllum hydrophobin genes. These data provide genetic evidence for the involvement of hydrophobins in the construction of the hydrophobic rodlet layer on the conidiospores. The results presented in this study suggest that these rodlet layers actually consist of self-assembled hydrophobins, excluding participation of any other substances in their formation, and that the hydrophobins themselves are responsible for the hydrophobicity of these rodlet layers.

With respect to morphogenesis in Schizophyllum, we assume that the presence of the hydrophobic rodlet layer at the hyphal surface is necessary for, or at least facilitates, the irreversible escape of hyphae from the hydrophilic substrate and their growth into the air, which typically has a low water potential (Wessels, 1993). Other hydrophobin genes (*Sc1* and *Sc4*) have also been identified in Schizophyllum (Schuren and Wessels, 1990), and the protein encoded by the *Sc4* gene has been identified (Wessels et al., 1991b). These genes are expressed at high levels in the dikaryon only, and the encoded hydrophobins have been implicated in fruit body formation (Wessels, 1993). The Sc4p hydrophobin also forms an insoluble complex through interfacial self-assembly and is present as such in the walls of hyphae within fruit bodies. However, the microscopic appearance of this hydrophobin complex is unknown, and its assumed role in adhesion of hyphae during tissue formation is speculative.

St. Leger et al. (1992) have cloned a hydrophobin gene from the insect pathogenic fungus *Metharizium anisopliae* and have speculated that the encoded protein plays a role in adherence of haustoria of the fungus to the insect cuticle. Recently, a hydrophobin gene was isolated from the rice blast fungus *Magnaporthe grisea*; this gene is highly expressed during appressorium formation, whereas the fungus shows decreased pathogenicity when the gene is disrupted (Talbot et al., 1993), again suggesting a role in adhesion. Although with the exception of the presumed wilting toxins and Sc3p, none of the hydrophobins have yet been purified and studied in detail, the genetic studies cited above reveal the variety of important roles that hydrophobins play in fungal morphogenesis and pathogenesis.

METHODS

Organism, Culture Conditions, and Labeling

The monokaryotic strain 4-40 (*A43B43*, CBS 340.81) and a derived *thin* mutant (Wessels et al., 1991a) were grown from a mycelial macerate on the surface of minimal medium (if necessary solidified with 1.5% agar) (Dons et al., 1979) at 25°C in the light. For the large-scale isolation of Sc3p hydrophobin, Fernbach flasks containing 250 mL of minimal medium were inoculated with 10 mL of a mycelial macerate of the wild-type monokaryon and grown for 6 days.

For the isolation of ^{35}S-labeled proteins, the wild-type monokaryon was grown in the presence of ^{35}S-sulfate as described by de Vries et al. (1986).

For small-scale experiments, the mycelium was separated from the medium by filtration through paper (Whatman No. 1). For large-scale isolations, the medium was collected by filtration through nylon gauze (first 200- and then 30-μm mesh) and then centrifuged (10,000*g* for 30 min) to remove any mycelial fragments. Because Sc3p aggregates by freezing, the clear culture medium was used directly for experimentation or purification of Sc3p.

To obtain hyphal material for electron microscopy, the fungus was grown for 5 days on a polycarbonate membrane (pore size 0.1 μm; Poretics, Livermore, CA) lying on solid medium. To make a distinction between submerged and aerial hyphae, the colony was overlayed with a second polycarbonate membrane that prevented formation of aerial hyphae. Aerial hyphae that grew through small holes punctured in the top membrane were collected.

For the isolation of wall-bound Sc3p hydrophobin, aerial hyphae were collected on a large scale by scraping them off frozen mycelial mats. Cell walls were isolated and treated with hot 2% SDS as described by Wessels et al. (1991a). They were then washed three times with 1% Nonidet P40 to remove any SDS, followed by washing with water, and freeze drying.

Purification of Sc3p

This method is based on the property of Sc3p to aggregate at water–air interfaces.

The medium from 5-day-old standing cultures of the wild-type monokaryon, cleared by centrifugation (10,000*g* for 30 min), was vigorously mixed with air in a blender running at full speed for 5 min and again centrifuged at 10,000*g* for 30 min. The pellet, resulting from aeration, was washed four times with water and freeze dried. The freeze-dried material (30 mg/L of culture medium) was taken up in 100% trifluoroacetic acid (TFA) (200 μL/mg of freeze-dried material) at 4°C. After centrifugation, the supernatant was taken to dryness in a stream of nitrogen. The dried material (1 mg/mL) was resuspended in 60% ethanol, 0.1 M Tris, pH 8.0, and left overnight at room temperature. The suspension was centrifuged at 10,000*g* for 30 min, and the supernatant

was dialyzed against three changes of water for 60 min each. The purified Sc3p was then precipitated by bubbling nitrogen through the retained solution.

In some cases, the freeze-dried material obtained after mixing the medium with air was extracted five times with chloroform/methanol (2:1 [v/v]) at 65°C for 10 min before proceeding with purification of Sc3p as described above.

To purify Sc3p from hot SDS–extracted walls of aerial hyphae, these were first subjected to a chloroform/methanol extraction (2:1 [v/v]) to remove noncovalently bound lipids. Sc3p was then extracted and dissociated by sonicating the walls in TFA (100 μL/mg) three times for 30 sec while keeping the suspension on ice.

Analytical Procedures

SDS-PAGE was performed on 12.5% (w/v) polyacrylamide gels according to the method of Laemmli (1970). Samples taken up in SDS sample buffer after treatments with TFA were adjusted to pH 6.8 with 1 M Tris. For nonradioactive proteins, a Phast System (Pharmacia, Bromma, Sweden) was used, and staining with silver was done in the development unit according to instructions of the manufacturer.

Conventional SDS-PAGE was used for radioactive proteins. Autoradiography was done by soaking the gel in 1 M sodium salicylate immediately after electrophoresis (Chamberlain, 1979). The gels were dried at 65°C and exposed to X-OMAT film (Kodak) at −80°C.

Electron Microscopy

For freeze-fracture and freeze-etch observations, the material was frozen in a mixture of solid and liquid nitrogen. Freeze etching and freeze fracturing were done either in a Bioetch 2005 (Leybold, Cologne, Germany) or a freeze-etch unit (Balzers, Liechtenstein). Replicas were made using Pt/C or Ta/W/C. All replicas were cleaned in $K_2Cr_2O_7$ saturated H_2SO_4 for 90 min.

Surface shadowing was done with Pt/C at an angle of 30° after drying the material on Formvar-coated nickel grids.

For negative staining, aggregated proteins were dried on carbon- and Formvar-coated nickel grids and stained with 1% aqueous uranyl acetate for 10 sec.

For thin sectioning, aggregated proteins were taken up in molten agar and fixed on ice for 60 min in 3% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.2, 0.2% ruthenium red. The fixed material was washed in buffer and postfixed with 0.67% OsO_4, 1.67% $K_2Cr_2O_7$ for 2 hr at room temperature. After washing with water, the material was incubated in 1% uranyl acetate overnight at room temperature. After dehydration in an ethanol series, the material was embedded in Epon 812 resin.

Materials were examined in an electron microscope (Philips CM 10; Eindhoven, The Netherlands). Photographs were made on FGP Kodak film.

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