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Involvement of Extracellular Matrix and Integrin-like Proteins on Conidial Adhesion and Appressorium Differentiation in *Magnaporthe oryzae*

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Abstract Conidial adhesion and appressorium formation of *Magnaporthe oryzae* on the rice surface are important early events in the infection process. As an initiative step to understand the mechanisms underlying these cellular processes at a biochemical level, the effect of a human fibronectin antibody (HFA) and RGD peptides on conidial adhesion and appressorium formation was evaluated. HFA inhibited conidial adhesion and appressorium formation in a dosage-dependent manner. RGD peptides also inhibited these cellular events. Conidial adhesion and appressorium formation inhibited by RGD peptides were restored by chemicals involved in the cyclic AMP-dependent signaling pathway. These results suggest that extracellular matrix proteins might be involved in conidial adhesion and appressorium formation through integrin-like receptor mediation and modulation of cAMP-dependent signaling in the cells.

Keywords: *Magnaporthe oryzae*, conidial adhesion, appressorium formation, extracellular matrix, fibronectin, integrin, signaling pathway

Magnaporthe oryzae is the causal fungus of rice blast, the most destructive diseases on rice worldwide. Successful infection by this fungus requires a series of steps including conidial adhesion, germination, appressorium formation, and penetration [33]. A number of environmental signals including surface hydrophobicity [19, 24], hardness of the contact surface [35], and chemical components of the plant surface [11] have been identified as major triggering factors in infection-related morphogenesis. Fungal genes involved in infection structure formation have also been isolated and characterized (reviewed in [33]). However, there is little information on the principle components involved in fungal cell adhesion to the host surface during infection.

Conidial adhesion is the earliest step in the infection process and important for initial communication between the fungal pathogen and host. Conidia of *M. oryzae* have mechanisms for immediate and persistent attachment to various surfaces by releasing spore tip mucilage (STM) from the conidial apex [13]. However, the biochemical properties of STM and the mechanism for adhesion are not clearly understood. In other plant pathogenic fungi such as *Colletotrichum* spp., conidial adhesion requires components of the extracellular matrix (ECM) [7]. The fungal ECM has been considered to have numerous functions including adhesion that prevents displacement from the infection point [7, 27]. However, the components of ECM released by ungerminated conidia are not well characterized. In mammalian systems, host fibronectin as an ECM adhesive protein has a role in cell adhesion by numerous pathogenic bacteria and yeast [12, 18, 22, 25, 31, 32].

Recognition and mediation of extracellular signals are *via* transmembrane glycoproteins known as integrins [15–17]. Integrins mediate cell adhesion and signal transduction in mammalian, yeast, and plant cells. Masking or ligation of the external integrin domain with antibodies or with the tripeptide Arg-Gly-Asp (RGD) inhibits functions of integrins [2, 28–30]. Integrins exhibit specific affinities to the tripeptide sequence Arg-Gly-Asp (RGD) found in several extracellular matrix components. Integrins are thought to be present and functional in budding yeast [14]. In other fungal species, a gene encoding integrin-like protein in *Candida albicans* was isolated and characterized [8, 9]. Integrin-like protein was detected in hyphal apices of *Saprolegina ferax* as patches associated with the plasma membrane using antibodies to a synthetic peptide of integrin from chicken embryo fibroblasts [20].

In the present work, we investigated the effect of a human fibronectin antibody (HFA) and several RGD-containing peptides on conidial adhesion and appressorium formation

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of *M. grisea* to explore the participation of fibronectin-like protein(s) and integrin-like protein(s) in the fungal infection process. We also speculated the involvement of integrin-like protein in the signal transduction pathway in the infection-related development of the pathogen.

MATERIALS AND METHODS

Fungal Strain and Cultural Conditions

Magnaporthe oryzae strain 70-15 was kindly provided by Dr. A. H. Elingboe at the University of Wisconsin, Madison, U.S.A., and used throughout the experiment. The fungus was grown on oatmeal agar medium (50 g of oatmeal in 1 l of water) at 25°C for 10–14 days under constant light to promote sporulation. Conidia were harvested and washed 3 times with distilled water. The concentration of conidial suspension was adjusted to 5×10^5 conidia/ml for experimental assay.

Conidial Adhesion Assay

Conidial adhesion was measured on the hydrophobic or hydrophilic surface of GelBond, a product of FMC (Rockland, ME, U.S.A.). Each 5 μl drops (5×10^5 conidia/ml) of conidial suspension and test solution was placed on the GelBond in a moistened plastic box. Total conidia were counted by direct microscopic examination after 2 h incubation at 25°C. Nonadhesive conidia were washed out with water for three times up-down. Then, only remained conidia were counted. The frequency of conidial adhesion was assessed by the numbers of retained conidia counted by microscopy in comparison with the total number of conidia before removing. It was calculated from the examination of about 200 conidia with three replicates. All experiments were repeated at least three times.

Conidial Germination and Appressorium Formation Assay

Conidial germination and appressorium formation were conducted on the hydrophobic side of the GelBond as described by Kim *et al.* [21] and Cho *et al.* [4]. Briefly, 5 μl drops of conidial suspension were placed on the GelBond, sealed, and incubated at 25°C for 2 h. When almost conidial adhesion occurred, then the same volume of chemical solution (2×) was added on the GelBond, and incubated for another 14 h. The percentage of conidia with appressoria was determined from direct microscopic examination of 200 germinated conidia with three replicates in three experiments.

Preparation of Chemicals

All chemicals used for these experiments were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.), unless otherwise indicated. The test chemicals were in proper solvents and made into stock solutions (2×), unless otherwise indicated. The RGD, RGDS, GRGDS, and GGGG peptides were purchased from BACHEM (Bubendorf, Switzerland) and other GRGD, GRGDSPK, and RGES peptides were purchased from Sigma-Aldrich Chemical Co. They were dissolved in sodium-phosphate buffer (10 mM, pH 5.8) and diluted [6]. The human fibronectin antibody was developed in a rabbit and affinity isolated. It was supplied as a solution in 0.01 M phosphate-buffered saline, pH 7.4, containing 1% bovine serum albumin and 0.1% sodium azide (MSDS) as a perservative. It was diluted from 1:50 to 1:250 with distilled water for the pharmacological experiment. Three complementation chemicals were used as follows: cAMP (cyclic adenosine 3,5-monophosphate) and 1,16-hexadecanediol (diol) were dissolved in distilled water. IBMX (3-isobutyl-methylxanthine) was dissolved in ethanol.

RESULTS AND DISCUSSION

Kinetics of Conidial Adhesion and Appressorium Formation of *M. oryzae*

After deposition of the conidial suspension on the surface, conidial adhesion occurred within 30 min and about 80% of conidia adhered to the hydrophobic surface of the GelBond within 90 min prior to germination. After this time, conidia began to germinate and the adhesion rate was further increased to its maximum level. Therefore, adhesion after this time point was considered as that of germinated conidia (Fig. 1). Conidial adhesion was recorded at 90 min after dropping the conidial suspension on the hydrophobic surface of the GelBond, throughout this study. Conidial germination occurred within 2 h, followed by swelling on the tips of most germ tubes. At about 6 h after incubation,

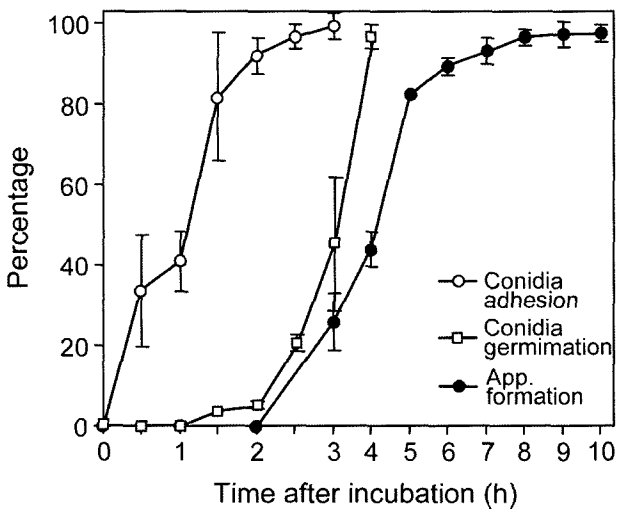

Fig. 1. Kinetics of conidial adhesion, germination, and appressorium formation of *M. oryzae* on the hydrophobic surface of a GelBond. Percentage of conidial adhesion and appressorium formation was determined by counting approximately 200 conidia. The data are the results of at least three experiments with three replicates in each.

dome-shaped appressoria began to appear, and at about the 8-h incubation period, darkly melanized, mature appressoria were observed on almost all of the germ tubes (Fig. 1). These data indicate that two phases of adhesion occur in this fungus. Immediate early adhesion of nongerminated conidia was almost completed within 2 h. This early adhesion may protect spores from displacement from the leaf surface before being germinated. After that time, firm attachment was associated with the growing germ tube, which is known to be essential for appressorium formation [19]. Adhesion of nongerminated conidia to various surfaces immediately upon hydration is known to be mediated by spore tip mucilage (STM) [13]. This immediate adhesion does not seem to accompany protein biosynthesis because sodium azide and cycloheximide did not affect conidial adhesion. The composition of STM is not yet fully defined, but high molecular weight glycoproteins containing α-mannoside or α-glucoside were thought to be involved [34]. These extracellular matrix glycoproteins (ECMs) are believed to mediate the sensing and relaying of extracellular signal for conidial adhesion and appressorium differentiation [34]. One ECM-encoding gene, *EMP1*, was isolated and characterized to be involved in conidial adhesion and appressorim development in *M. oryzae* [1]. In the *Δemp1* mutant, appressorium formation was reduced to about half of its wild-type strain, which was recovered by the addition of IBMX, a cyclic AMP (cAMP) phosphodiesterase inhibitor. These data suggest that EMP1 relays extracellular signals into fungal cells *via* the cAMP-mediated signal transduction pathway, although the receptor accepting the EMP1 signal is not identified yet [1]. In addition, we found that autoclaved conidia can adhere to the surface, suggesting that heat-stable nonproteinous materials are also involved in conidial adhesion (data not shown).

In mammalian cells, signaling across a dynamic continuum from the ECM to the inside of the cell is manifested *via* interactions between plasma membrane-bound receptors known as integrins and protein ligands within the ECM that contain Arg-Gly-Asp (RGD) motifs [5, 10]. Those ECM proteins with RGD motifs include fibronectin, laminin, vitronectin, and collagen. Integrins are connected to intracellular proteins that include diverse signaling molecules enriched at focal adhesion (or contacts) and are also linked to the actin cytoskeleton [3]. This linear linkage from the ECM outside of a cell to the actin cytoskeleton *via* integrin receptors is essential for an efficient signaling connection for cells to respond to extracellular cues. We tested the involvement of integrin signaling in pre-infection morphogenesis in the rice blast fungus.

Effect of Human Fibronectin Antibody on Conidial Adhesion and Appressorium Formation

We previously observed that human vitronectin antibody co-localized with the conidial apex and along the germ tube and appressorium (unpublished data), suggesting the involvement of ECM proteins on conidial germination and appressorium formation. We tested the function of fibronectin, a major component of ECM proteins, on these developmental processes using human fibronectin antibody (HFA). HFA in a 100-fold diluted concentration significantly inhibited conidial adhesion and appressorium formation. The inhibition showed a dosage-dependent manner (Fig. 2A). However, HFA did not affect conidial germination and germling growth. These observations led us to the hypothesis that a fibronectin-like protein(s) in the ECM of *M. oryzae* is engaged in the process of sensing and/or signal transmission for conidial adhesion and appressorium formation. Adhesion to host cells through binding to fibronectin, thereby forming a biofilm, has been

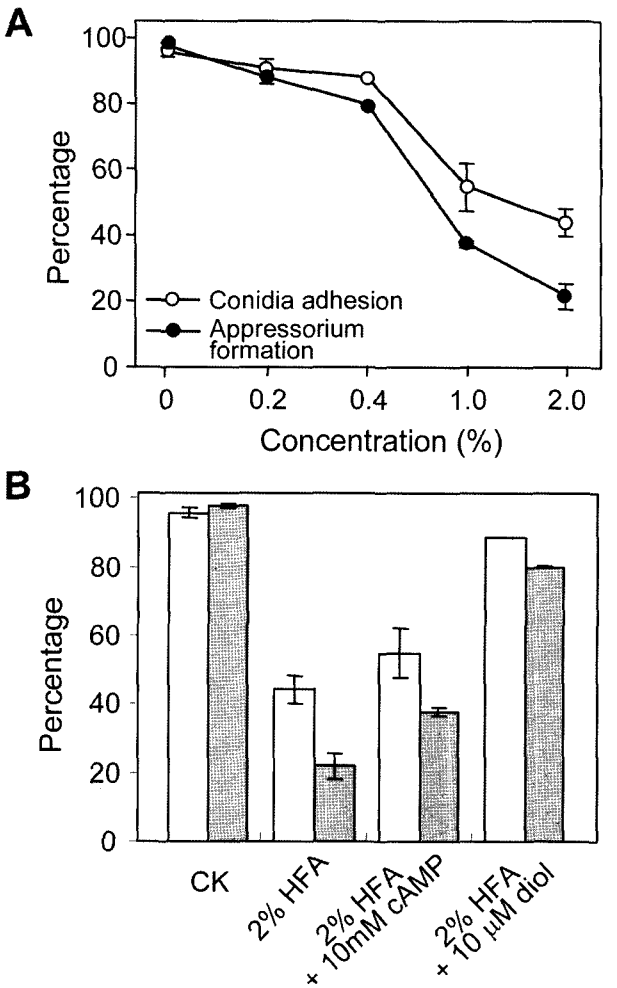

Fig. 2. Effect of HFA on the conidial adhesion and appressorium formation of *M. oryzae*.

A. Dosage-dependent inhibition of cellular processes. **B.** Chemical complementation of the HFA effect by cAMP and 1,16-hexadecanediol. Open bar, conidial adhesion; gray bar, appressorium formation. Experiments were conducted on the hydrophobic surface of a GelBond. The data are the result of at least three experiments with three replicates in each.

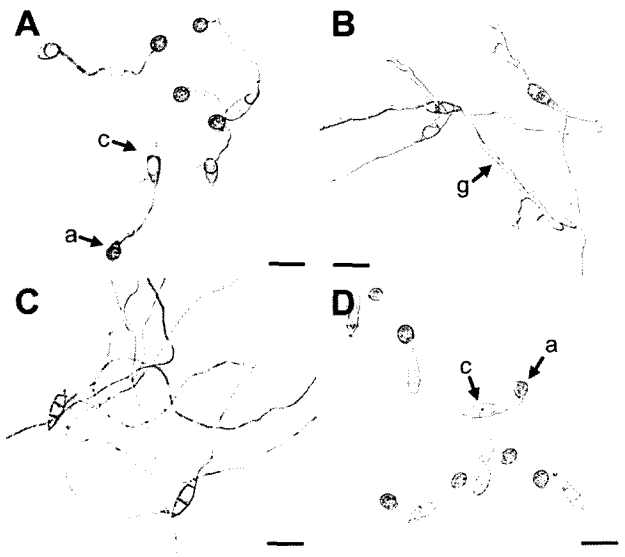

Fig. 3. Appressorium formation of *M. oryzae*.
A. Appressoria formed on the hydrophobic surface of a GelBond. **B.** Germinating conidia on the hydrophilic surface of a GelBond. **C.** Inhibition of appressorium formation on the hydrophobic surface by 2% HFA. **D.** Restoration of appressorium formation inhibited by HFA by the addition of cAMP. The bar in each panel indicates 20 μm. a, appressorium; c, conidia; g, germ tube.

considered as a major step in infection of *C. albicans* [22, 26].

The involvement of possible signal transduction pathways was also tested by the addition of cAMP and 1,16-hexadecanediol (a cutin monomer). The cutin monomer restored the conidial adhesion and appressorium formation inhibited by 2% HFA to more than 80%. cAMP also recovered these developmental processes, although the effect was not that obvious. Chemically restored appressoria appeared normal in shape and were well melanized upon maturation (Fig. 3). These results suggest that the cAMP-mediated signaling cascade is involved in relaying signals transferred from fibronectin in *M. oryzae*. Signals responding to the cutin monomer, a cell wall component, were also sufficient to nullify the inhibitory effect of HFA.

Effect of RGD-containing Peptides on Conidial Adhesion and Appressorium Formation

The inhibitory effects of synthetic RGD-containing peptides on conidial adhesion and appressorium formation were tested. Conidial germination and germling growth were not affected in the presence of RGD-sequence-containing peptides (RSCP). Conidial adhesion and appressorium formation were inhibited when conidia were incubated in the presence of RGD, RGDS, RGES, and to a lesser extent in a GRGDSPK. However, GRGD, GRGDS, and GGGG did not affect these developments (Table 1). Among the peptides tested, RGD was the most effective in inhibiting

Table 1. Effect of RGD-sequence-containing peptides on conidial adhesion and appressorium formation by *M. oryzae*.

Treatment[a]	Conidial adhesion[b]	Appressorium formation[b]
RGD	47.1±8.4	23.4±8.8
RGDS	54.5±15.9	19.8±5.1
RGES	54.7±8.8	22.6±7.5
GRGD	73.3±5.1	77.4±7.3
GRGDS	63.9±10.7	68.4±12.2
GRGDSPK	63.0±5.5	35.8±8.0
GGGG	85.3±7.3	78.2±5.2
Control[c]	78.7±5.6	80.7±5.8

[a]The final concentration of each peptide was adjusted to 2 mM.
[b]Data are of mean and standard deviation.
[c]The phosphate buffer (10 mM, pH 5.8) alone was used as a control.
Experiments were conducted on the hydrophobic surface of a GelBond. Data show the results of three experiments with three replicates in each.

conidial adhesion, and RGDS in appressorium formation, respectively, in *M. oryzae*. Inhibition of appressorium formation was apparent by RGD in the concentrations of 0.5 to 2.0 mM in a dose-dependent manner, whereas inhibition of conidial adhesion occurred at the concentration above 2 mM (Fig. 4A). These data suggest that integrin-related proteins are involved in the conidial adhesion and appressorium development of *M. oryzae*. The involvement of integrin in the mediation of cell adhesion and signal transduction has been widely known in mammalian, plant, and yeast species. RSCP inhibited the attachment of *C. albicans* blastospores to endothelial cells, thereby reducing tissue invasion in murine infection [22, 23]. The involvement of integrin-like protein in fungal morphogenesis was also assessed in the bean rust fungus, *Uromyces appendiculatus* [6]. Each RSCP seemed to have species-specific and/or common effects on fungal development. RSCP was not effective in inhibiting conidial germination in *M. oryzae*, whereas GRG, GRGDS, and GRGDSPK affected urediospore germination of *U. appendiculatus*, both in their germination rate and germling morphology during early germination [6]. Changes in germling morphology and growth were not observed in *M. oryzae*. RGD, RGDS, and GRGDSPK were effective in appressorium formation in both species, whereas GRGD and RGES showed specific effects. These common and specific effects might be attributed to the diversity of integrin-like proteins among fungal species. Alternatively, it could involve a different structural conformation of the ligand and the receptor site characteristics to particular species.

Possible signal transduction pathways mediating integrin signals in conidial adhesion and appressorium formation were assessed by the treatment of cAMP, IBMX, and 1,16-hexadecanediol, all of which restored both developmental processes inhibited by RGD (Fig. 4B). These results give indirect evidence for the involvement of integrin-related signaling with cAMP- and cutin monomer-dependent signal transduction pathways. The fact that

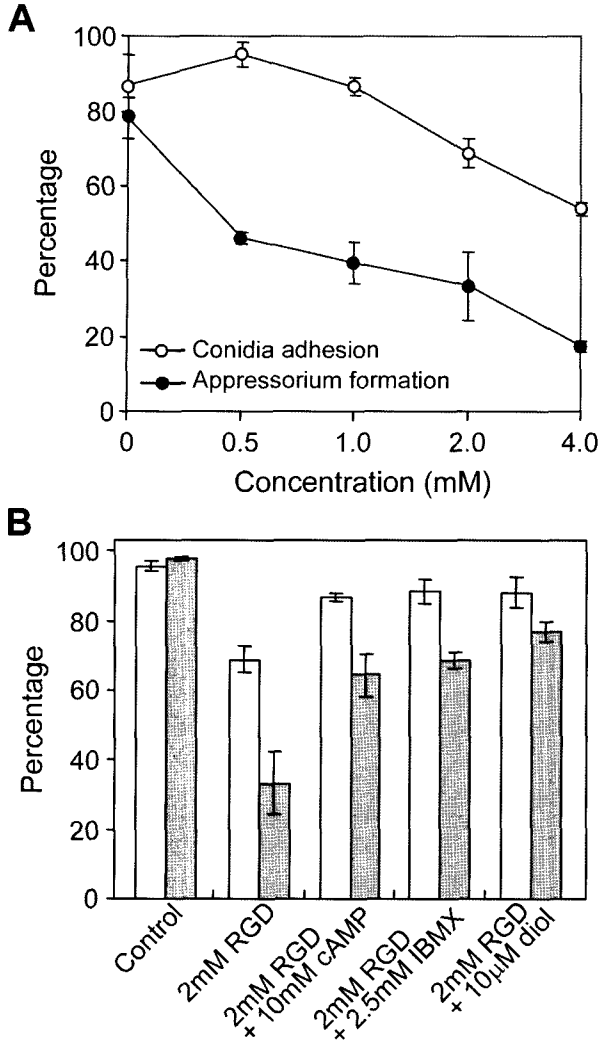

Fig. 4. Effect of RGD peptide on conidial adhesion and appressorium formation by *M. oryzae*.
A. Dosage-dependent inhibition of conidial adhesion and appressorium formation. **B.** Chemical complementation of the HFA effect by cAMP, IBMX, and 1,16-hexadecanediol. A control treatment was only phosphate buffer (10 mM, pH 5.8) of same volume instead of RGD peptide. Open bar, conidial adhesion; gray bar, appressorium formation. Experiments were conducted on the hydrophobic surface of a GelBond. The data are the result of at least three experiments with three replicates in each.

IBMX, an inhibitor of cAMP phosphodiesterase, as well as exogenously added cAMP could restore conidial adhesion and appressorium formation inhibited by RGD suggested that both the synthesis and degradation of cAMP are important in relaying the signals transmitted through integrin-like proteins in *M. oryzae*.

In conclusion, we have shown here that extracellular matrix protein such as fibronectin and membrane spanning receptor integrin are involved in conidial adhesion and appressorium formation, the initial two steps for plant infection, in *M. oryzae*. Furthermore, signals transmitted to fibronectin-like and integrin-like proteins might be relayed through the cAMP-dependent signal transduction pathway within the fungal cells. Understanding the precise biochemical mechanisms involved in conidial adhesion and appressorium formation is not only of biological interest but novel strategy to control this plant disease.

Acknowledgments

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