The *RIM101* Signal Transduction Pathway Regulates *Candida albicans* Virulence during Experimental Keratomycosis

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PURPOSE. To examine the role of the fungal *RIM101* signal transduction pathway in the pathogenesis of *Candida albicans* keratitis.

METHODS. *C. albicans* wild-type strain SC5314, prototrophic mutant control DAY185, and homozygous fungal mutants for the *rim8*, *rim13*, *rim20*, *rim101*, and *phr1* genes were evaluated in vitro using proliferation and filamentation assays. Scarified corneas of BALB/c and C57BL/6J mice were topically inoculated and observed daily for keratitis severity. Corneal adaptation and pathogenicity were assessed ex vivo by maintaining infected porcine corneas for 3 days in an explantation culture system for histologic evaluation of hyphal penetration.

RESULTS. All *C. albicans* strains had similar growth kinetics, and SC5314 and DAY185 demonstrated pH-induced filamentation. Fungal mutants had reduced hyphal formation at alkaline and neutral pH, but normal acidic assays ascertained that mutant strains did not have a generalized filamentation defect. SC5314 and DAY185 caused moderate to severe keratitis in mice, whereas fungal strains lacking constituents of the *RIM101* pathway had significantly ($P < 0.05$) attenuated severity in vivo. Three days after inoculation of porcine corneas, SC5314 and DAY185 produced hyphae that penetrated 28% and 25%, respectively, of the corneal thickness, and all five mutant strains showed significantly ($P < 0.05$) less stromal penetration.

CONCLUSIONS. The *RIM101* signal transduction pathway plays an important role in the development of *C. albicans* keratitis. The fungal pathway intermediates Rim8p, Rim13p, Rim20p, and Rim101p and the downstream cell-wall protein Phr1p are pivotal in the process of corneal invasion by *C. albicans*. (*Invest Ophthalmol Vis Sci.* 2010;51:4668–4676) DOI:10.1167/iovs.09-4726

The fungus *Candida albicans* causes systemic mycosis, opportunistic mucosal candidiasis, endogenous endophthalmitis, and suppurative keratitis.[1–4] Attributes of *C. albicans* the contribute to adaptation and tissue invasion include the secretion of degradative enzymes and the ability to switch from yeasts to filamentous forms.[5] *C. albicans* keratitis occurs when, in response to conditions at the ocular surface, blastospores transition from saprophytic commensals into invasive microorganisms. After attachment to the injured cornea, pseudohyphae and hyphae invade the corneal stroma.[6]

Morphologic versatility is a distinctive feature of dimorphic fungi such as *Candida* species. Specifically, the pleomorphic nature of *C. albicans* allows a range of growth patterns from yeasts to filaments that develop as apical extensions from blastospores.[7,8] Pseudohyphae and hyphae facilitate attachment and invasiveness, properties associated with fungal virulence for host tissues and the pathogenic process.

C. albicans uses a set of conserved pathways to regulate its morphogenic state, including the *RIM101* pathway, the mitogen-activated protein (MAP) kinase pathway, a cAMP-dependent protein kinase pathway, the *CZF1* matrix pathway, and Tup1-mediated repression.[9] When environmental cues are sensed by fungi, the transduction pathway specific for a particular signal is activated, resulting in corresponding transcription factors that induce fungal genes to produce proteins enabling hyphal filamentation.[10–13]

Ambient pH influences fungal filamentous growth through a conserved pathway first identified in *Saccharomyces cerevisiae* and *C. albicans*,[14,15] with a homologous cascade found in ascomycetes and basidiomycetes.[16–19] Named the *RIM101* pathway or *PacC* pathway, depending on the fungal genus, a key intermediate is the zinc finger–containing transcription factor Rim101p/PacC.[19–23] Under acidic conditions Rim101p/PacC is full-length and inactive. However, at neutral to alkaline conditions its C-terminal portion is cleaved, resulting in activation of the protein subunit that alters gene expression.[24–27] In the *RIM101* signal transduction pathway, Rim13p is responsible for the proteolytic activation of the transcription factor Rim101p.[15,20]

Rim101p contributes to virulence in models of disseminated candidiasis.[15,22,28] Previous studies suggest that the pH responsive *RIM101* pathway may also be involved in the pathogenesis of fungal corneal infection. For example, in a model of experimental murine keratitis, an insertional mutation of *rim13* results in attenuated virulence of *C. albicans*.[6,29] In addition, the transcription factor Efg1p, a key component of several signal transduction pathways including the *RIM101* pathway, is pivotal for filamentous growth of *C. albicans* during the establishment of keratomycosis. A lack of *efg1* diminishes corneal virulence, whereas a reintegrant is invasive and virulent.[30,31]

Based on this evidence, we hypothesized that Rim101p affects virulence in the experimental keratomycosis model. This current investigation examined the role of the *RIM101* signal transduction pathway in the pathogenesis of keratomycosis. Using genetic knockout mutants of *C. albicans*, we confirmed the need for Rim13p and determined the importance of pathway components Rim8p, Rim20p, and Rim101p. We also studied whether corneal virulence depends on Phr1p,

a pH-regulated protein with transcription that is governed by Rim101p and is involved in assembly of the fungal cell wall during filamentous growth at neutral and alkaline conditions.[15] Using models of in vivo murine keratomycosis and ex vivo porcine corneal infection we showed that these components of the *RIM101* pathway allow *C. albicans* to infect the cornea.

MATERIALS AND METHODS

Fungal Strains

A wild-type strain (SC5314) of *C. albicans*, originally isolated from human infection; a prototrophic mutant control reference strain (DAY185); a homozygous *Tn7* transposon mutant (GK088); and four engineered homozygous deletion mutants (DAY25, DAY111, DAY117, and CAS10) were evaluated (Table 1). Strain SC5314 has been used extensively for genetic studies of *C. albicans* and experimentally causes corneal disease in rabbits and mice.[29,32,33] The prototrophic mutant control reference strain DAY185 was created by transformation of strain BWP17 and has a wild-type Ura+Arg+His+ genotype.[28] Strain GK088 (Tn7-rim13) is a homozygous mutant with a transposon insertion at position 239 of the *rim13* coding sequence[20,29] that was transformed into the SC5314-derivative strain BWP17[19] as part of a homozygous insertion mutant library.[34] The mutant strains DAY25, DAY111, and DAY117 were generated and described by PCR-directed gene knockout by using consecutive transformation of strain BWP17.[19,28] The mutant strain CAS10 was generated by homozygous deletion of *phr1* from the SC5314 derivative CAF3–1.[35]

Corneal inocula were prepared by culturing yeasts on Sabouraud dextrose agar (Difco, Detroit, MI) for 3 days at 25°C. Colonies were harvested and diluted in sterile phosphate-buffered saline (PBS) to yield 2×10^4 colony-forming units (CFU)/μL. A 5-μL aliquot was applied to the right eyes.

In Vitro Growth Kinetics

Yeast strains were grown in 1% yeast extract, 2% peptone, and 2% dextrose (YPD) liquid medium at 30°C overnight and then harvested and suspended in sterile phosphate-buffered saline (PBS). Optical density (OD) was measured with a spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Princeton, NJ) at a wavelength of 600 nm (OD_{600}). A conversion factor of one OD_{600} unit equivalent to 3×10^7 colony-forming units (CFU)/mL was used to estimate fungal concentration.[36,37] Triplicate samples of 3×10^5 CFU for each strain were inoculated into 25 mL M199 liquid media (Invitrogen, Grand Island, NY) at pH 6.0, 7.3, and 8.0 and incubated at 27°C with a shaking speed of 150 rpm. *C. albicans* concentrations were determined spectrophotometrically at 1.5, 3, 4.5, 6, 9, 12, 15, and 24 hours postinoculation

(PI). The doubling time for each strain at each pH was calculated from the slope of the linear regression equation describing the exponential growth phase. Student's *t*-test was used for pairwise comparisons, and $P < 0.05$ was considered statistically significant.

Filamentation Assays

For pH-induced filamentation assays, yeast strains were grown on Sabouraud dextrose agar for 2 days at room temperature then harvested, diluted in sterile PBS, and inoculated onto M199 agar plates containing Earle's salts and glutamine but lacking sodium bicarbonate.[6,38] The plates were buffered with 2 M Tris-HCl to yield media of pH 4.0, 7.3, and 8.0. Inoculated plates were incubated at 37°C, and colonies were observed daily by inverted microscopy for 7 days. Filamentation was scored by the pace of onset, according to the postinoculation day that hyphae/pseudohyphae were first observable using a procedure as previously described.[6] The degree of filamentation based on the number and length of filaments was also noted.

Animals and Experimental Keratomycosis

Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Female BALB/c and C57BL/6J mice 6 to 8 weeks of age (Harlan Sprague-Dawley, Houston, TX) were anesthetized intraperitoneally with ketamine, xylazine, and acepromazine. The corneas of right eyes were superficially scarified as previously described.[37] A 5-μL inoculum of *C. albicans* (1×10^5 CFU) was topically applied to right eyes (five mice per group). Mice were monitored with a dissecting microscope daily for up to 7 days PI, to determine the severity of keratomycosis by criteria that assigned grades of 0 to 4 for inflammatory area, density, and surface irregularity.[6,37] Findings were documented by slit lamp photomicrography.

Ex Vivo Testing

All seven strains of *C. albicans* were evaluated in porcine corneas by using an explantation model.[39] Fresh porcine eyes were obtained from Visiontech, Inc. (Mesquite, TX), after immersion in a saline solution of penicillin, streptomycin, and amphotericin B (Invitrogen, Grand Island, NY). An 18-mm diameter trephine (VisionPak, Lexington, KY) was used to excise corneas that were then fixated onto an artificial chamber (Refractive Technologies, Cleveland, OH). Superficial scarification with a 22-gauge needle was used to produce a 15×15 cross-hatch pattern, and 10 μL containing 1×10^5 CFU of the *C. albicans* strains was topically applied to each cornea (three corneas per fungal strain). Inoculated corneas were placed into a six-well culture dish

TABLE 1. Genotypes, Phenotypes, and the Effects of pH Variation on Growth Rates for *C. albicans* Strains

|			Doubling Time (h)*		
Strain	**Genotype**	**Phenotype**	**pH 6.0**	**pH 7.3**	**pH 8.0**
SC5314[32]	Clinical isolate	Wild-type	1.17 ± 0.02	1.28 ± 0.02	1.45 ± 0.01
DAY185[28]	*ura3Δ::λimm434* *pHIS1::his1::hisG* *arg4::URA3::arg4::hisG* *ura3Δ::λimm434* *his1::hisG* *arg4::hisG*	Wild-type	1.54 ± 0.02	1.46 ± 0.02	1.61 ± 0.06
DAY25[28]	*ura3Δ::λimm434* *pHIS1::his1::hisG* *arg4::hisG rim101::URA3* *ura3Δ::λimm434* *his1::hisG* *arg4::hisG rim101::ARG4*	Rim101p-negative	1.46 ± 0.02	1.66 ± 0.01	1.71 ± 0.04
DAY111[19]	*ura3Δ::λimm434* *his1::hisG arg4::hisG rim20::ARG4 pRIM101* *ura3Δ::λimm434* *his1::hisG arg4::hisG rim20::URA3 RIM101*	Rim20p-negative	1.50 ± 0.03	1.45 ± 0.02	1.83 ± 0.03
DAY117[28]	*ura3Δ::λimm434* *HIS1::his1::hisG* *arg4::hisG* *rim8::ARG4* *ura3Δ::λimm434* *his1::hisG* *arg4::hisG* *rim8::URA3*	Rim8p-negative	1.48 ± 0.02	1.46 ± 0.02	1.86 ± 0.02
GK088[20]	*ura3Δ::limm434* *his1::hisG* *arg4::hisG* *rim13::URA3* *ura3Δ::limm434* *his1::hisG* *arg4::hisG* *rim13::ARG4*	Rim13p-negative	1.44 ± 0.10	1.56 ± 0.06	1.97 ± 0.03
CAS10[35]	*Δura3::imm434* *Δphr1::hisG* *Δura3::imm434* *Δphr1*	Phr1p-negative	1.24 ± 0.02	1.59 ± 0.02	2.00 ± 0.03

* Mean ± SD.
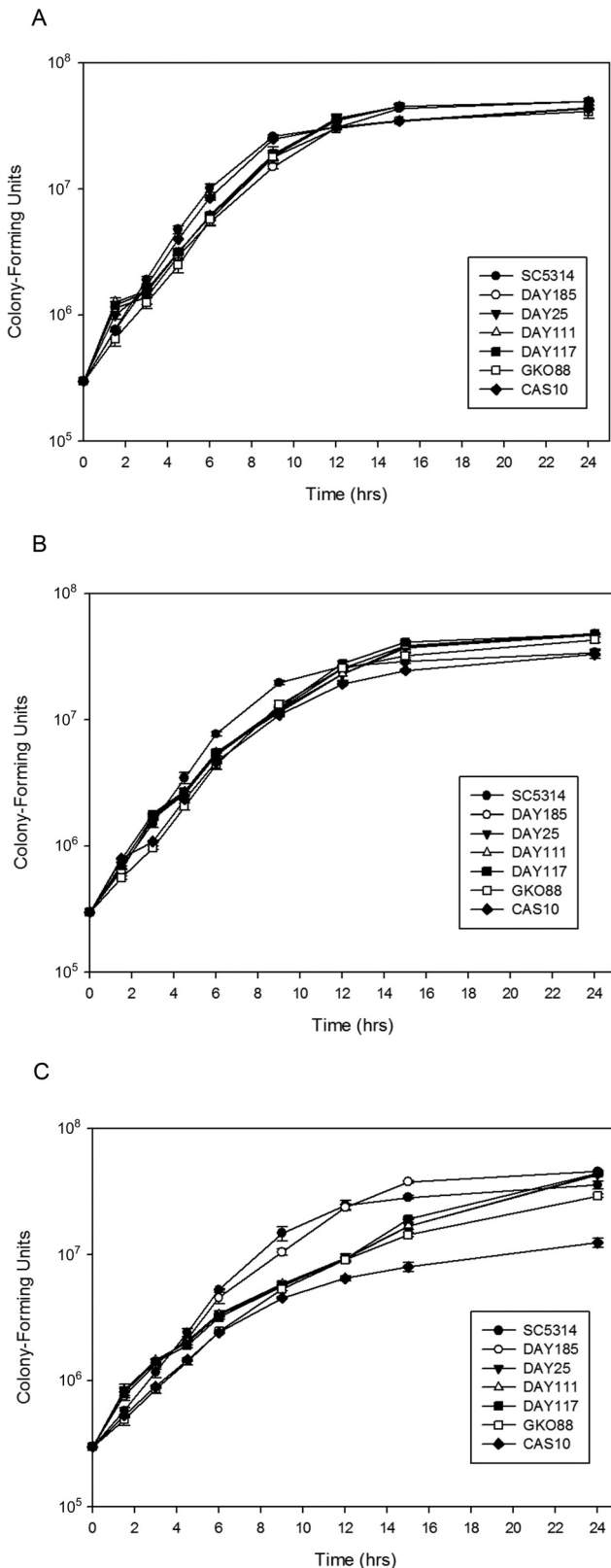
Figure 1. In vitro growth kinetics of *Candida albicans* strains SC5314, DAY185, DAY25, DAY111, DAY117, GKO88, and CAS10. Triplicate samples of each strain were inoculated into M199 media at pH 6.0 (**A**), 7.3 (**B**), and 8.0 (**C**) and monitored by spectrophotometry over time. Mean OD_{600} measurements converted to colony-forming unit equivalents ($\pm$ SD) are plotted.

(Corning, Corning, NY) so that the periphery was immersed in modified supplemented hormonal epithelial medium (SHEM).[39] Tissues were incubated at 34°C in 5% CO_2 with 95% humidity, and the SHEM was changed daily. After 72 hours, the corneas were embedded in OCT compound (Sakura Finetec, Torrance, CA) and frozen at -80°C for subsequent histopathologic processing.

Hyphal Penetration

The average maximum hyphal penetration of porcine corneas was determined as previously described.[39] Ten-micrometer frozen sections were cut and stained with periodic acid-Schiff (PAS) reagent (Sigma-Aldrich, St. Louis, MO). Three sections were examined for each cornea. Images were obtained of an entire limbus-to-limbus region for each corneal section by using a digital camera attached to a microscope (Y-FL; Nikon, Tokyo, Japan). Hyphal penetration depth and total corneal thickness were measured with an image analysis system (NIS-Element 3.0; Nikon). The maximum hyphal penetration was estimated from measurements taken at five regions of each corneal section demonstrating the greatest depth of corneal penetration. Results from the three areas with deepest penetration were averaged for each histologic section. The absolute maximum penetration depth and the percentage of penetration relative to corneal thickness at the points measured were recorded. Results were compared for statistical significance by Student's *t*-test.

RESULTS

In Vitro Comparison of *C. albicans* Strains

Since an important consideration in studying microbial virulence is the overall growth ability of the organism, all *C. albicans* strains used in this study were evaluated with acidic, neutral, and alkaline media to determine whether any inherent growth advantages or disadvantages existed. Although there were absolute growth differences among the strains that varied in different pH conditions, strains SC5314, DAY185, DAY25, DAY111, DAY117, GKO88, and CAS10 demonstrated similar lag, log growth, and plateau phases (Fig. 1), with strain SC5314 tending to grow more rapidly than other strains ($P < 0.05$). All strains demonstrated a trend for reduced growth rates as pH increased (Table 1), resulting in a significant difference between pH 6.0 and 8.0 ($P < 0.05$). Consistent with the removal of alkaline-response genes, the mutant strains were more affected by increased pH conditions with respect to growth rates than SC5315 or the prototrophic strain DAY185 (Fig. 1C).

Further characterization of the fungal strains involved in vitro evaluation of filamentation phenotype. The relative filamentation results are shown in Table 2. *C. albicans* produced hyphae and pseudohyphae on artificial media, and filamentous growth was enhanced as the culture media pH increased. At neutral and alkaline pH conditions the wild-type strain SC5314 and the prototrophic strain DAY185 demonstrated rapid filamentation: obvious filaments were observed on day 1 PI and progressed through-

TABLE 2. In Vitro Filamentation of *C. albicans* Strains

Strain	pH 4.0	pH 7.3	pH 8.0
SC5314	+ +	+ + +	+ + +
DAY185	+ +	+ + +	+ + +
DAY25	+ +	+	+
DAY111	+ +	+	+
DAY117	+ +	+	+
GKO88	+ +	0	0
CAS10	+ +	0	0

+ + +, filamentation initially observed 1 day PI; + +, filamentation initially observed 3 days PI; +, filamentation initially observed 7 days PI; 0, no filamentation observed by 7 days PI.

out 7 days of observation. Strains deficient in genes of the *RIM101* alkaline response pathway demonstrated greatly reduced filamentation phenotypes at neutral and alkaline conditions based on the time required for hyphae or pseudohyphae to initially present as well as the overall number and length of the filaments. In contrast to the wild-type and prototrophic controls, the mutant strains demonstrated little to no hyphal production at pH 7.3 and 8.0, even at 7 days PI. Consistent with the pathway mutations being evaluated, filamentation was not affected under acidic conditions (Table 2).

In Vivo Experimental Fungal Keratitis

Scarified corneas of BALB/c mice inoculated with different strains of *C. albicans* were evaluated to determine the relative virulence of the homozygous mutants in vivo. The severity of corneal disease was scored daily for 7 days and assigned a categorical score of 0 to 12 as previously described.[37] An inoculation of 1×10^5 CFU of the wild-type strain SC5314 and the prototrophic control strain DAY185 caused moderate corneal disease, whereas the five mutant strains were attenuated in relative virulence (Fig. 2). Representative findings for each strain at 1, 3, and 7 days PI are shown in Figure 3. SC5314 produced more severe disease with an average score of 7.3 ± 1.2 on day 1 PI and a mean score of 5.3 ± 0.6 on day 7 PI. Strain DAY185 produced slightly less corneal disease than SC5314, with mean scores of 6.8 ± 1.1 at 1 day PI and 3.2 ± 2.2 at 7 days PI, but the differences were not significantly different from the scores of SC5314 at any point during the study period ($P > 0.05$). Eyes infected with the mutant strains had mean disease severity scores ranging from 0.6 ± 0.6 to 2.3 ± 0.6 at day 1 PI depending on the particular strain. By day 7 PI, all indication of infection and corneal disease had resolved in the fungal mutant-infected BALB/c mice, resulting in null scores. Statistical comparison demonstrated no significant difference in disease severity among the mutant strains ($P > 0.05$) at any day other than GK088 at day 1 PI. Comparison of the mutants to SC53114 and DAY185 revealed a significant difference ($P < 0.05$) for each day of observation.

Previous work demonstrated that experimental fungal keratitis can be induced in both inbred strains and outbred stocks of mice.[37] To extend these findings and to uncover additional insight into the role of the *RIM101* signal transduction pathway in corneal disease, scarified corneas of C57Bl/6J mice were inoculated with 1×10^5 CFU of SC5314, GK088, or CAS10. Eyes were evaluated and scored daily for 7 days (Fig. 4). Representative results for 1, 3, and 7 days PI are shown in Figure 5. As in BALB/c mice, SC5314 induced severe disease that was significantly greater than the disease caused by either GK088 or CAS10 ($P < 0.005$). SC5314 and GK008 caused greater disease in C57BL/6J mice than in BALB/c mice with mean scores 2 to 3 units higher for each time point evaluated. CAS10 did not produce measurable inflammatory disease in either BALB/c or C57BL/6J mice.

Ex Vivo Keratomycosis in Porcine Corneas

Porcine corneas maintained in an explantation culture system were inoculated with 1×10^5 CFU of SC5314, DAY185, DAY25, DAY111, DAY117, GK088, or CAS10 (three corneas per fungal strain). Three days PI corneas were cryosectioned and histopathologically evaluated. Representative results are shown in Figure 6. Extensive epithelial sloughing and abundant fungi in the corneal stroma were seen in SC5314- and DAY185-infected corneas. Hyphae and pseudohyphae were the predominant fungal forms. Fungal mutant-inoculated corneas showed significantly less fungal burden with fewer hyphae and pseudohyphae within the stroma. Although some epithelial thickening was observed, the epithelial layer was largely intact in mutant-infected corneas.

The extent of hyphal penetration was quantified by measuring the maximum penetration depth for each experimental group (Table 3). SC5314 and DAY185 showed equivalent maximum penetration as determined by absolute penetration (791.7 ± 235.4 μm and 743.7 ± 209.1 μm, respectively) and by penetration relative to the corneal thickness at the point of penetration (27.8% ± 10.9% and 25.0% ± 7.0%, respectively). No significant difference in penetration ability was seen between these two strains with respect to either absolute ($P = 0.93$) or relative penetration ($P = 0.73$). The five mutant strains each demonstrated less than half the average maximum penetration of SC5214 and DAY185, measured by absolute or relative penetration (Table 3). Histologic data demonstrated that the mutant strains were significantly less invasive than the wild-type and prototrophic control strains ($P < 0.05$).

DISCUSSION

Sequencing of the *C. albicans* genome and development of techniques for targeting specific genes have led to a research upsurge into investigating how particular fungal genes contribute to the pathogenesis of candidiasis.[13,40,41] Selective mutants have helped to identify key intermediates affecting fungal survival and pathogenicity. Stemming from ophthalmic studies on the morphogenesis and pathogenicity of *C. albicans*,[6,29,32,37] we used a systematic gene-deletion approach to examine how fungal keratitis entails genetically regulated mechanisms enabling the formation of invasive hyphae into the corneal stroma.

Pathways affecting the morphologic plasticity of *C. albicans* are interactive and interdependent.[5,7–13] Environmental cues are detected by the microorganism resulting in intracellular signaling that culminates in responses at the gene level via various transcription factors. Signals known to elicit the transition of *C. albicans* blastospores into hyphal forms include temperature and ambient pH.[42] Conditions at the ocular surface, such as a temperature of 34°C and a pH of 7.5,[43–45] favor hyphal growth of *C. albicans*.[46,47]

Several gene products are necessary for pH sensing and signaling, and the molecular process has been reviewed in detail.[5,7–11,48] In brief, surface receptor proteins Dfg16p and
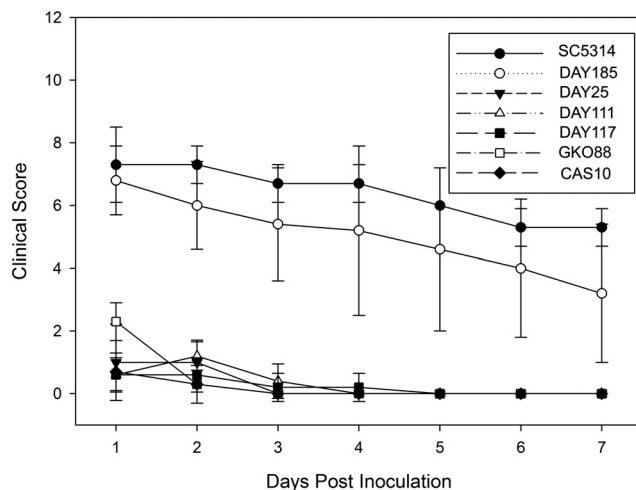

FIGURE 2. Murine keratomycosis severity induced by *C. albicans* in BALB/c mice. Immunocompetent BALB/c mice were infected with 10⁵ CFU of *C. albicans* used in the study and were followed and scored for disease severity for 7 days. Each point represents the mean score (± SD) of five eyes.
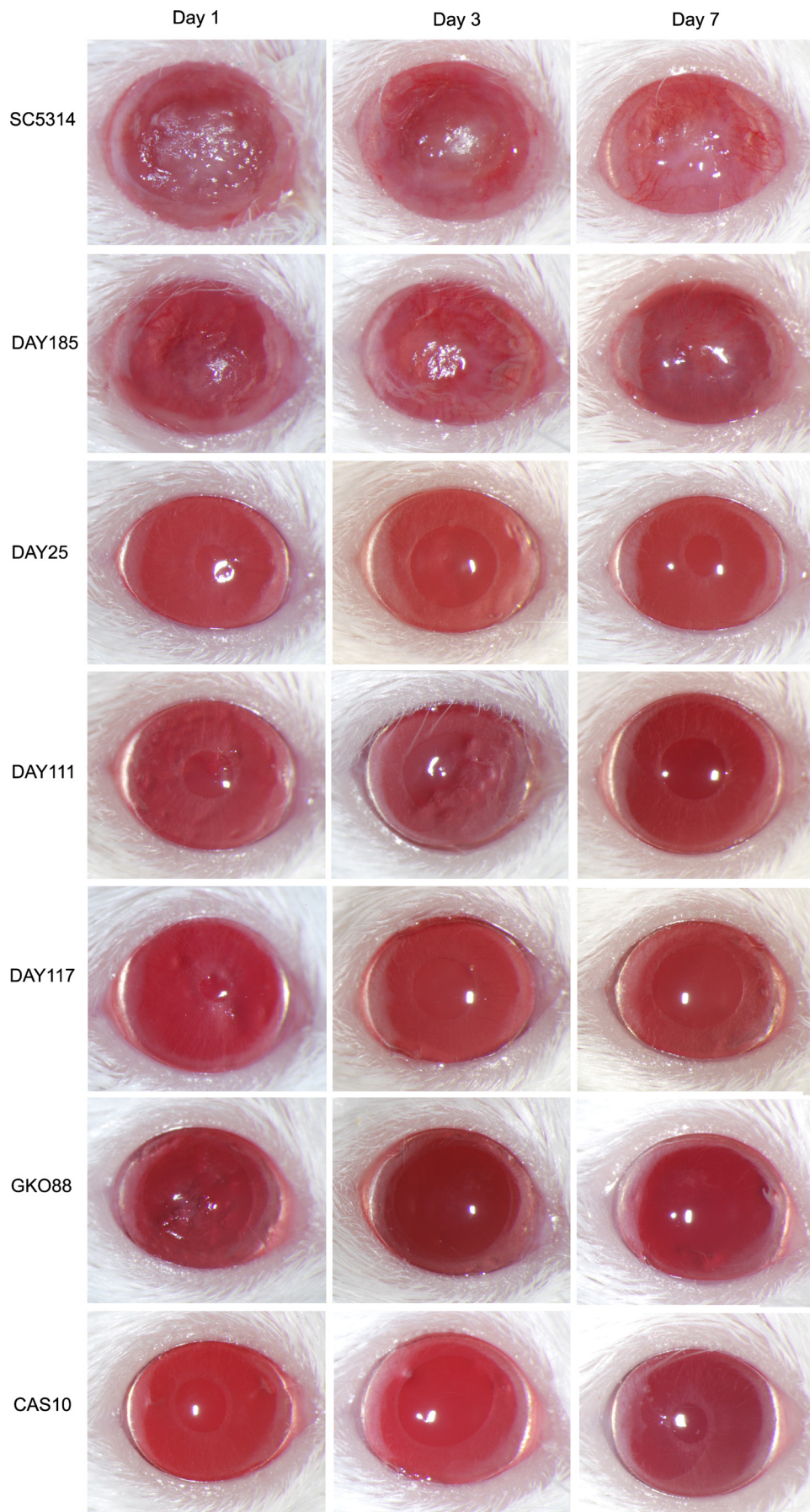
FIGURE 3. Progression of murine keratomycosis induced by *C. albicans* in BALB/c mice. Scarified corneas of immunocompetent BALB/c mice were infected with 10^5 CFU of *C. albicans* used in the study and photographed on days 1, 3, and 7 PI. Representative images among five eyes per group are shown.
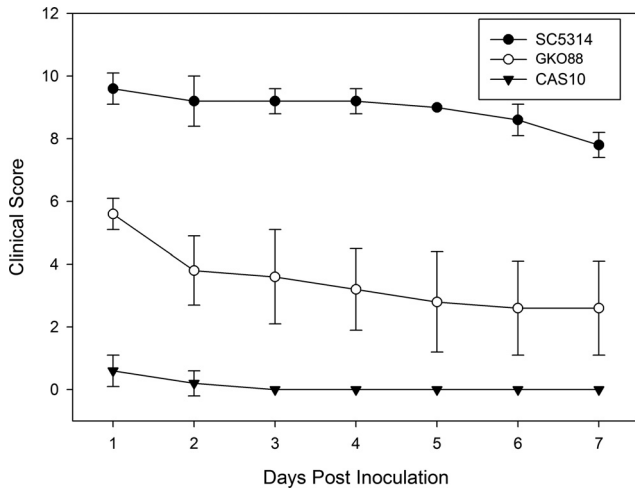
Figure 4. Murine keratomycosis severity induced by *C. albicans* in C57BL/6J mice. Immunocompetent C57BL/6J mice were infected with 10^5 CFU of *C. albicans* strains SC5314, GKO88, or CAS10 and were scored for disease severity for 7 days. Each point represents the mean score (± SD) of five eyes.

Rim21p/PalH detect environmental pH, and Rim9p/PalI assists in the localization of Rim21p/PalH to the plasma membrane.[49–52] Stimulation of the membrane sensor prompts ubiquitination and promotes endocytosis of the Rim8p/PalF protein that is associated with Rim21p/PalH.[53] This interplay results in the recruitment of endosomal sorting complexes that include Vps20-Snf7p/Vps32 proteins.[54] Snf7p/Vps32 oligomerizes on the endosome and recruits Rim20p/PalA and the calpain-like protease Rim13p/PalB.[20,54,55] Rim20p/PalA binds to the inhibitory domain on the carboxyl end of the full-length, inactive transcription factor Rim101p/PacC.[56] Rim13p/PalB has proteolytic activity and removes the inhibitory C-terminal domain of

Rim101p/PacC. This processed, active form translocates into the nucleus to regulate transcriptional changes promoting pH-dependent responses.[38,57,58]

The present study confirms our previous observation that a $rim13^{-/-}$ mutant of *C. albicans* has significantly reduced corneal virulence.[29] Compared with wild-type *C. albicans*, the *rim13*-null mutant had minimal filamentation ability on explanted corneas and failed to establish progressive corneal infection in vivo. We also examined the role of other components of the conserved *RIM101* signal transduction pathway. Although the proteolytic activity of Rim13p or a previously unknown biological activity independent of the *RIM101* pathway cannot be excluded as mechanisms influencing fungal invasion, our observations imply that the *RIM101* signal transduction pathway regulates the development of *C. albicans* keratitis.

Rim101p is one of the principal regulators of host–pathogen interactions, as exemplified by a $rim101^{-/-}$ strain demonstrating a severe virulence defect in a murine model of systemic candidiasis.[28] Similarly, based on previous studies demonstrating a correlation between clinical appearance and histopathology in a murine model of experimental keratomycosis,[29,37,59] our current results show that this regulatory transcription factor is a determinant of virulence in corneal infection. The $rim101^{-/-}$ homozygous mutant strain DAY25 induced only mild corneal inflammation without obvious infection, and mice recovered fully within 1 to 2 days. Furthermore, in explanted corneas the $rim101^{-/-}$ mutant strain only invaded the superficial corneal layer compared with deep stromal penetration by the wild-type and prototrophic strains. These in vivo and ex vivo findings provide evidence implicating Rim101p in mediating *C. albicans* virulence. Together with our previous studies of a Rim13p-deficient mutant, these results implicate the *RIM101* signal transduction pathway as being directly involved in the pathogenesis of *C. albicans* keratitis.
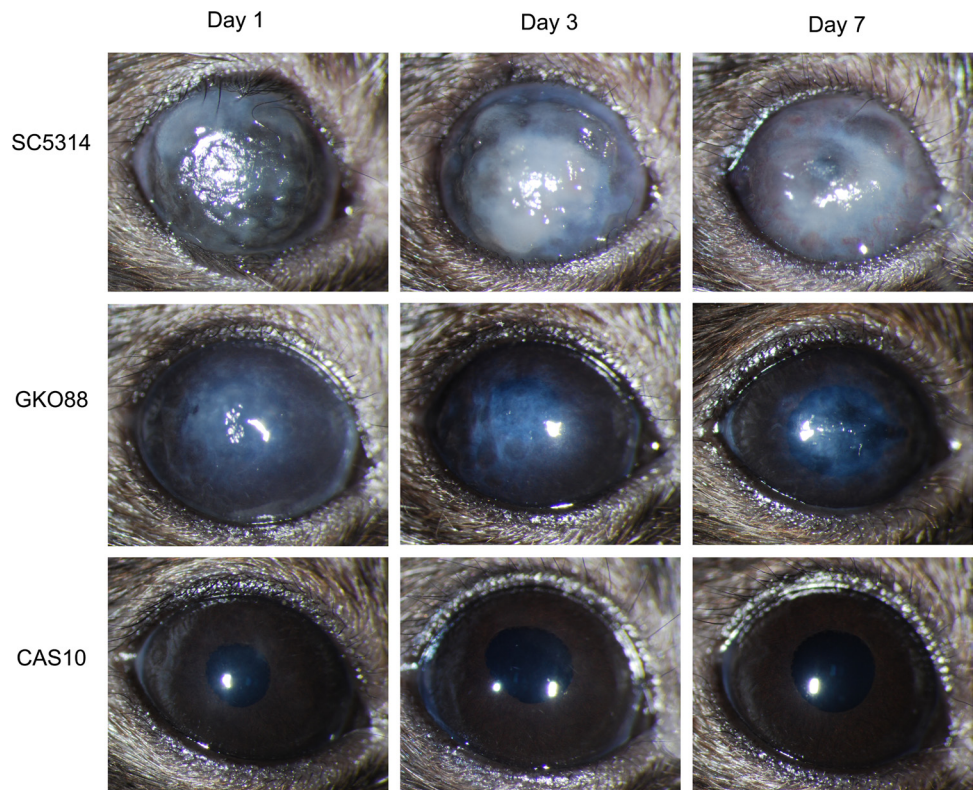

Figure 5. Progression of murine keratomycosis induced by *C. albicans* in C57BL/6J mice. Scarified corneas of immunocompetent C57BL/6J mice were infected with 10^5 CFU of *C. albicans* strains SC5314, GKO88, or CAS10 and were photographed on days 1, 3, and 7 PI. Representative images among five eyes per group are shown.
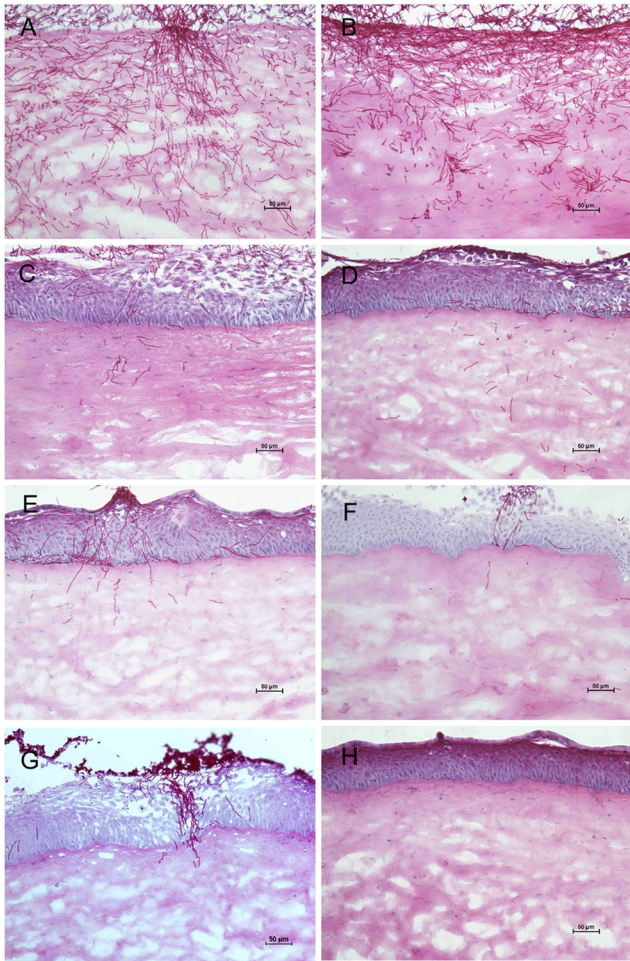
FIGURE 6. Histopathology of porcine keratomycosis induced by *C. albicans* strains. Porcine corneas inoculated ex vivo with 10^5 CFU of *C. albicans* strains SC5314 (**A**), DAY185 (**B**), DAY25 (**C**), DAY111 (**D**), DAY117 (**E**), GKO88 (**F**), or CAS10 (**G**) or with PBS (**H**) were cryosectioned at 10-μm thickness 3 days PI and stained with periodic acid-Schiff. Representative sections among three corneas per strain are shown. Original magnification ×200.

Other constituents of the *RIM101* pathway including Rim20p and Rim8p have been identified in *C. albicans*,[19] and our results confirmed that these molecules are also required for corneal virulence. These findings demonstrate that several components leading to the activation of Rim101p affect *C. albicans* virulence during corneal infection. In comparison, restoration of virulence to the prototrophic mutant control DAY185 confirms the effects of selective gene disruption and shows that attenuation of the fungal mutants was not due to a nonspecific consequence of the molecular techniques used to generate the genetic deletions. The highly conserved nature of the *RIM101/PacC* signal transduction pathway across fungal species further suggests that these genes or their respective homologues have corresponding roles in virulence for other fungi.

We also examined a downstream product of Rim101p activation. Activated Rim101p directly and indirectly regulates the expression of alkaline pH-induced genes that are involved in fungal filamentation.[15] In particular, the *phr1* gene is a target of the *RIM101* pathway that is involved in the yeast-to-hypha transition. This gene encodes a glycosylphosphatidylinositol-anchored cell surface glycosidase that cross-links carbohydrate polymers needed for integrity of the fungal cell wall.[60] A *phr1*-null mutant is unable to undergo apical growth at neutral-to-alkaline pH and has reduced virulence for producing systemic infection in BALB/c mice.[35,61] Strain CAS10, the *phr1*$^{-/-}$ mutant that we used in the present study, did not produce filamentous forms in explanted porcine corneas and was avirulent in the immunocompetent murine cornea. Furthermore, in a parallel study, we found that the relative regulation of *phr1* under alkaline conditions is significantly lower in VE175, a wild-type strain of *C. albicans* having attenuated corneal virulence and altered in vitro morphology compared with the virulent SC5314 strain.[39] *Phr2*, a functional homologue of *phr1* that is preferentially expressed at acidic pH and repressed at alkaline pH in a Rim101p-dependent fashion,[19,62] was significantly upregulated in the hypovirulent VE175 strain.[39] Thus, our findings are consistent with other recent studies on the role of the *RIM101* pathway and its downstream targets in fungal pathogenicity.

This investigation indicates that the *RIM101* pathway is important for corneal virulence by *C. albicans*, but other virulence mechanisms may also be involved. Mds3p is necessary for growth and hyphal formation at alkaline pH, independent of the *RIM101* pathway,[27,34] and is involved in virulence in both a mouse systemic model[34] and the ocular model.[6] Sla2p is an actin-binding protein necessary for alkaline pH–induced hyphal formation.[63,64] Sch9p is a serine-threonine protein kinase needed for embedded hyphal growth.[27] Suv3p is an ATP-dependent RNA helicase involved in mitochondrial RNA catabolism, embedded filamentation, and growth under oxygen-limited conditions.[27] Sap6p is one of 10 known secreted aspartyl proteinases and can influence virulence through several possible mechanisms, but its effect on corneal virulence is likely associated with filamentation.[31] Interrelated pathways affect fungal morphogenesis as well as pathogenicity.

In addition to the mycological processes of candidal infection, the murine models used in this study indicate that host

TABLE 3. Maximum Penetration of *C. albicans* Strains into Porcine Corneal Stroma

Strain	Penetration Depth* (μm)	P†	Penetration Ratio‡ (%)	P†
SC5314	761.7 ± 235.4	0.93	27.8 ± 10.9	0.73
DAY185	743.7 ± 209.1	Referent	25.0 ± 7.0	Referent
DAY25	168.5 ± 85.7	0.01	4.9 ± 1.9	0.009
DAY111	295.1 ± 79.7	0.03	9.4 ± 2.2	0.02
DAY117	340.3 ± 144.8	0.03	11.1 ± 1.6	0.03
GK088	207.9 ± 110.6	0.02	9.7 ± 2.9	0.03
CAS10	55.4 ± 62.2	0.005	2.7 ± 3.6	0.008

* *C. albicans* maximum infiltrating depth, mean ± SD.
† Pairwise comparison of each strain to prototrophic strain DAY185.
‡ Mean maximum penetration depth relative to corneal thickness, % ± SD.

factors modulate the severity of keratomycosis. We found that C57BL/6J and BALB/c mice had inherent differences in susceptibility to *C. albicans* and developed disparate levels of severity of fungal keratitis. Although both mouse strains were vulnerable to corneal infection, C57BL/6J mice tended to develop more severe disease. This interstrain difference is similar to reports for models of bacterial keratitis in which C57BL/6 mice were more susceptible to *Pseudomonas aeruginosa* infection than BALB/c mice.[65] In addition, we previously reported a difference between inbred BALB/c mice and outbred NIH Swiss mice with respect to *C. albicans* keratitis.[37] These findings disclose an opportunity to explore how host resistance contributes to the susceptibility and severity of fungal keratitis.

In summary, model systems and molecular biological techniques provide the means to dissect the mechanisms of infectious eye disease. This study indicates that pathogens respond to local conditions at the ocular surface and provides insight into the molecular machinery directing fungal infection of the cornea. Ongoing research into the pathogenesis of keratomycosis offers prospects for translational studies on improved control and prevention.

Acknowledgments

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