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Reduced expression of the hyphal-independent *Candida albicans* proteinase genes *SAP1* and *SAP3* in the *efg1* mutant is associated with attenuated virulence during infection of oral epithelium

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The transition of *Candida albicans* from a yeast to a hyphal form is controlled by several transcriptional factors, including the key regulators Cph1 and Efg1, and is considered an important virulence attribute. These factors, especially Efg1, regulate the expression of hyphal-associated genes e.g. *SAP4*–*SAP6*. In order to investigate the relevance of these transcriptional regulators for hyphal-independent *SAP* genes, recently constructed *cph1* and *efg1* single mutants and a *cph1*/*efg1* double mutant lacking these factors were tested during interaction with oral epithelium and polymorphonuclear neutrophils. In contrast to the parental wild-type strain and the *cph1* mutant, the *efg1* and the *cph1*/*efg1* mutants did not produce hyphal forms in all experiments and were less capable of damaging epithelial cells and neutrophil granulocytes. The attenuated epithelial lesions of these mutants were correlated not only with reduced expression of the hyphal-associated gene *SAP4*, but also with the lack of *SAP1* and *SAP3* expression previously shown to be important for oral infections. An *efg1* mutant strain carrying a plasmid-borne copy of the *EFG1* gene regained hyphal growth, damage of keratinocytes, granulocytes and the expression of *SAP1* and *SAP3*. Although *efg1* and *cph1*/*efg1* mutants did not produce germ tubes during infection, expression of the hyphal-associated genes *SAP5* and *SAP6* was not completely abolished. A reduced capacity to stimulate an epithelial immune response manifested by a delayed onset of IL-1β, IL-8 and TNF expression was only observed in the *cph1*/*efg1*-infected tissue. These results provide further evidence for a combined regulation of different virulence factors, such as dimorphism and expression of *SAP* genes. Furthermore, it could be demonstrated that the lack of Efg1 also caused reduced expression of hyphal-independent *SAP* genes. Both the *EFG1* and the *CPH1* gene products are necessary for adequate induction of an immune response.

INTRODUCTION

The yeast-to-hyphal transition (dimorphism) is known to be one of the most important virulence factors of the fungal pathogen *Candida albicans* (Brown & Gow, 1999; Calderone & Fonzi, 2001; Ernst, 2000). Dimorphism is regulated by different cross-talking signal transduction pathways. Efg1 (Stoldt *et al*., 1997; Bockmuhl & Ernst, 2001) and Cph1 (Bockmuhl & Ernst, 2001) have been identified as key transcriptional regulators for the cAMP-protein kinase (PKA) and the mitogen-activated protein kinase (MAPK) pathway, respectively. The role of these factors in morphogenesis and their relevance for *Candida* infections was studied using strains which lacked either functional Efg1, Cph1 or both factors (Brown & Gow, 1999; Stoldt *et al*., 1997; Bockmuhl & Ernst, 2001; Dieterich *et al*., 2002; Kohler & Fink, 1996; Leberer *et al*., 1997; Lo *et al*., 1997; Sonneborn *et al*., 1999b; Weide & Ernst, 1999; Lewis *et al*., 2002; Phan *et al*., 2000). The results obtained with these mutants suggested that disruption of *CPH1* caused attenuated hyphal formation abilities on solid media while mutants lacking *EFG1* failed to produce hyphal cells under most conditions investigated. Furthermore, mutants lacking *EFG1* showed a significantly attenuated virulence phenotype in a mouse model of systemic infection (Lo *et al*., 1997), in the interaction with macrophages (Lo *et al*., 1997), in the ability to colonize

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PMNs, polymorphonuclear neutrophils; RHE, reconstituted human oral epithelium.

successfully on polyurethane central venous catheters (Lewis *et al.*, 2002) and in the capacity to invade or injure endothelial (Phan *et al.*, 2000), epidermal or intestinal (Dieterich *et al.*, 2002) cells. However, under laboratory conditions of microaerophilic growth (Sonneborn *et al.*, 1999a) and using an alternative animal model (Riggle *et al.*, 1999), the *cph1/efg1* double mutant was still able to form filaments and to produce invasive lesions in the tongue of the infected germ-free piglets (Riggle *et al.*, 1999).

For oral candidosis, epithelial cells and polymorphonuclear neutrophils (PMNs) are the first line of host defence (Eversole *et al.*, 1997; Challacombe, 1994; Farah *et al.*, 2001). We therefore investigated the interaction of the *cph1*, *efg1* and *cph1/efg1* mutants, their parental wild-type strain SC5314 and an *EFG1* revertant with reconstituted human oral epithelium (RHE) and with PMNs. Previously we have shown that certain secreted aspartyl proteinases (Saps) contribute to virulence in this model (Schaller *et al.*, 1998, 1999) and that the mucosal immune response is modulated differentially by different *Candida* species depending on their virulence potential (Schaller *et al.*, 2002). Recently it has been shown that Efg1 is involved in the regulation of both hyphal formation and expression of hyphal-associated *SAP* genes (Schröppel *et al.*, 2000; Staib *et al.*, 2002; Felk *et al.*, 2002). In the present study, we focused on the consequence of *EFG1* and *CPH1* disruption on the expression of hyphal-independent *SAP* genes and the induction of the immunomodulatory response by the hyphal-deficient mutants during infection of oral epithelium in comparison with wild-type cells.

METHODS

***Candida* strains, culture media and growth conditions.** For this study, the clinical *C. albicans* wild-type isolate designated SC5314 (Gillum *et al.*, 1984), the single mutants *cph1* (Lo *et al.*, 1997) and *efg1* (Stoldt *et al.*, 1997), the double mutant *cph1/efg1* (Lo *et al.*, 1997) and an *EFG1* reconstituted strain (Lo *et al.*, 1997) were used. For the incubation of *C. albicans* with PMNs and for the infection of the reconstituted epithelium, inocula were prepared as reported previously (Schaller *et al.*, 1998, 1999).

Reconstituted human epithelium. The reconstituted human epithelium was supplied by Skinethic Laboratory (Nice, France). Epithelial cells were seeded on inert filter substrates to form multi-layered oral epithelium. Cultures were incubated in serum-free conditions in a defined medium based on the MCDB-153 medium (Clonetics). This fully defined nutrient medium feeds the basal cells through the filter substratum. The medium was changed every 24 h. Three replicate infection experiments were performed with the *C. albicans* wild-type SC5314, the single mutants *cph1* and *efg1*, the double mutant *cph1/efg1* and the *EFG1* revertant. Epithelia were infected with 2×10^6 *C. albicans* yeast cells of each strain in 50 µl PBS for 12, 36, 46 and 52 h as described previously (Schaller *et al.*, 1998, 1999). Histological lesions of the samples caused by *C. albicans* were investigated by light microscopy. The *C. albicans*-induced epithelial damage was quantified by a lactate dehydrogenase (LDH) release assay, the expression of *SAP* genes and the epithelial immune response was studied by RT-PCR and the analysis of Sap protein secretion was performed by immunoelectron microscopy (see below).

Light microscopy. Light microscopical studies were performed as previously described (Schaller *et al.*, 1998, 1999) to evaluate histological changes during infection. A part of each specimen was fixed, postfixed and embedded in glycide ether. The small blocks of tissue were cut using an ultra-microtome (Ultracut; Reichert). Semi-thin sections (1 µm) were studied with a light microscope after staining with 1 % toluidine blue and 1 % pyronine G (Merck). The histological changes of the skin were evaluated on the basis of 50 sections from five different sites for each infected epithelium.

Assay of LDH activity. The release of LDH from epithelial cells into the surrounding medium was monitored as a measure of epithelial cell damage. LDH release in the maintenance media of the cultures from uninfected and infected epithelial cells was measured at 12 and 36 h. The LDH activity was analysed spectrophotometrically by measuring the NADH disappearance rate at 340 nm as a main wavelength during the LDH-catalysed conversion of pyruvate to lactate according to the Wróblewski-La Due method (Wróblewski & John, 1955). The LDH activity is expressed as U l^{-1} at 37 °C.

RNA isolation, cDNA synthesis (RT), PCR and pairs of primers. RT-PCR was used for analysis of *SAP* and cytokine gene expression during epithelial infection. Details of RNA preparation and cDNA synthesis have been published previously (Felk *et al.*, 2002). For an internal mRNA control and for detection of genomic DNA, we used *EFB1* (Maneu *et al.*, 1996). The DNA amplification fragment contained a 364 bp intron. Absence of genomic DNA was verified by a single PCR product of 564 bp. For detection of *EFB1* and *SAP1–10* transcripts specific pairs of primers were used (Table 1). The specificity of each set of primers was confirmed using genomic DNA. A similar sensitivity of the primer pairs for DNA amplification was determined by testing dilutions of genomic DNA. The cDNA samples were subjected to 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 60 °C, and extension for 1 min at 72 °C, and 10 min at 72 °C (final).

The measurement of epithelial cell cytokine gene expression was studied with specific primers for IL-1β, IL-8 and TNF (Table 1) by RT-PCR in uninfected RHE and 12, 36, 46 and 52 h after infection with *C. albicans* parental and mutant strains. Primers for amplification of the constitutive expressed genes encoding aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls (Table 1). The PCR conditions were 35 cycles of 1 min at 95 °C, 1 min at 65 °C, 1 min at 72 °C, and 5 min at 72 °C (final).

Immunoelectron microscopy. Post-embedding immunogold labelling was carried out as previously described (Schaller *et al.*, 1998, 1999) for intracellular detection of Sap antigen in epithelial samples infected with SC5314, *cph1*, *efg1*, *cph1/efg1* and the *EFG1* revertant taken after 12 and 36 h. After fixation, specimens were embedded in LR-White. Sections, 80–100 nm thick, were mounted on nickel grids. Grids were then incubated with anti-Sap polyclonal rabbit antibodies, directed against Sap1–3 (Borg-von Zepelin *et al.*, 1998). After washing overnight with PBS, grids were incubated with 10-nm-gold-conjugated goat anti-rabbit IgG (Auroprobe EM Immunogold reagents; Amersham). Grids were then fixed with 2 % glutaraldehyde and stained with 0·5 % uranyl acetate for 10 min and 2·7 % lead citrate for 5 min (Ultrastainer; LKB) at 20 °C. For examination of gold particles a Zeiss EM 902 transmission electron microscope was used operating at 80 kV, at magnifications between ×3000 and ×85 000. To back up the data statistically, we counted gold particles of 21 randomly chosen cells from each strain.

Isolation of PMNs. Neutrophils obtained from normal human volunteers were isolated from heparinized whole blood using the Histopaque-1119 in combination with Histopaque-1077 (Sigma) solution. The cells were suspended at a concentration of 5×10^7 ml^{-1} in RPMI 1640 medium (Sigma) containing 10 % FCS. Residual erythrocytes were removed by hypotonic lysis. Typical morphology of the

Table 1. *SAP*, *EFB1* and cytokine PCR primers used in this study and expected fragment length

Gene/cytokine	Expected fragment length (bp)	Primer sequence
SAP1	783	Fwd 5′-GAT GTC ATT AAA ACT CCT GTT AAT G-3′ Rev 5′-CCA GTT TCA ATT CAG CTT GG-3′
SAP2	915	Fwd 5′-CTC CTA AAG CAT TCC CAG TTA C-3′ Rev 5′-CAT CAT CAC CTT GTA AAG AAG C-3′
SAP3	802	Fwd 5′-CAT GTC AAG CTG GTC AAG GAC-3′ Rev 5′-ATA GGC TGA TCT CAA GAA ATT ATC-3′
SAP4	870	Fwd 5′-GTT CCA GAT TCA AAT GCC G-3′ Rev 5′-CTT GAG CCA TGG AGA TCT TTC-3′
SAP5	902	Fwd 5′-TGA GAC TGG TAG AGA TGG TG-3′ Rev 5′-GGT TTA CCA CTA GTG TAA TAT GT-3′
SAP6	605	Fwd 5′-AAA CCA ACG AAG CTA CCA GAA C-3′ Rev 5′-TAA CTT GAG CCA TGG AGA TTT TC-3′
SAP7	866	Fwd 5′-GAT AAG GCA TCA GGT ACT ATG G-3′ Rev 5′-AGG AAC AAC GGC ATG GTT ATC-3′
SAP8	903	Fwd 5′-CTG TTA TTG TTG ACA CAG GTT C-3′ Rev 5′-GTA GAA ATA CTT GAA GAA GTA GTG-3′
SAP9	898	Fwd 5′-CAC CAT AAG CAA CGT GAC TG-3′ Rev 5′-GCG AAA GCA ACA ACC CAT AC-3′
SAP10	938	Fwd 5′-ACG TCA GAA GAC TTT TCC ATT G-3′ Rev 5′-ATA TGG CGA TCC ATG AAC GTG-3′
EFB1	564/928 cDNA/gDNA	Fwd 5′-AGT CAT TGA ACG AAT TCT TGG CTG-3′ Rev 5′-TTC TTC AAC AGC AGC TTG TAA GTC-3′
TNF	236	Fwd 5′-GAGCTGAGAGATAACCAGCTGGTG-3′ Rev 5′-CAGATAGATGGGCTCATACCAGGG-3′
IL-1β	431	Fwd 5′-CGATCACTGAACTGCACGCTCCG-3′ Rev 5′-GGTGAAGTCAGTTATATCCTGGCCG-3′
IL-8	364	Fwd 5′-GCAGCTCTGTGTGAAGGTGCAG-3′ Rev 5′-GCATCTGGCAACCCTACAACAG-3′
Aldolase	572	Fwd 5′-AGCTGTCTGACATCGCTCACCG-3′ Rev 5′-CACATACTGGCAGCGCTTCAAG-3′
GAPDH	637	Fwd 5′-GCACCACCAACTGCTTAGCACC-3′ Rev 5′-GTCTGAGTGTGGCAGGGACTC-3′

PMNs was proved after Giemsa staining with light microscopy to ensure that a pure population of neutrophils (>95 % purity) had been isolated. The cells were vital-stained using the trypan blue dye exclusion method. Numbers of vital and non-vital leukocytes per sample were assessed using a Neubauer chamber. Viability of 98 % could be demonstrated.

Incubation of *C. albicans* with PMNs. A suspension of 4×10^7 cells of the wild-type isolate SC5314, the mutants *cph1*, *efg1* and *cph1*/*efg1* and the *EFG1* revertant in PBS was incubated for 150 min at 37 °C with 3×10^6 neutrophils to study the interaction of the different strains with PMNs by conventional electron microscopy and to evaluate the inhibitory effect of PMNs on yeast cell growth by a killing assay (see below).

Conventional electron microscopy. Electron microscopy was used to study the interaction of SC5314, *cph1*, *efg1*, *cph1*/*efg1* and the *EFG1* revertant with PMNs. Specimens were fixed in a phosphate-buffered solution (0·05 M, pH 7·3) with 2·5 % glutaraldehyde and 2 % formaldehyde following standard methods. Postfixation was based on 1 % osmium tetroxide in 0·1 M phosphate buffer at pH 7·3 at room temperature; the specimens were embedded in glycide ether. Ultrathin sections, 60–90 nm thick, were mounted on uncoated copper grids and stained in 2 % uranyl acetate for 30 min, then in Reynold's lead citrate for 8 min, and examined using a Zeiss EM 902 transmission electron microscope. To support our data statistically, we counted the number of *C. albicans* cells ingested by the PMNs and the number of obviously damaged fungal cells and leukocytes for each experiment by analysis of 30 representative electron microscopical figures.

Killing assay. A killing assay was used to study the inhibitory effect of PMNs on growth of the different *C. albicans* strains. It was performed by plating the *C. albicans* parental, mutant and revertant samples 150 min after incubation with and without PMNs on Sabouraud's dextrose agar. Before plating, samples were vigorously agitated by using a Pasteur pipette to separate big clumps of fungal cells into single cells and diluted 1 : 1000 and 1 : 10 000 in PBS. The presence of single cells was proved by light microscopy. Yeast cell viability was determined by assessment of the c.f.u. produced after incubation for 24 h at 37 °C on Sabouraud's dextrose agar. Three independent experiments were performed for each strain with and without PMNs.

Reproducibility and verification of the results. In order to examine the reproducibility of the histological alterations caused by the

C. albicans strains during RHE infection and interaction with PMNs, all experiments were carried out in triplicate.

RT-PCR analysis of *SAP* gene expression during infection of RHE *in vitro* was done in duplicate from each sample. Similar levels of cDNA in the experiments were verified by amplification of transcripts from housekeeping genes as internal controls. In all experiments, levels of amplification products for the genes encoding aldolase and GAPDH (keratinocytes) and *EFB1* (*C. albicans*) were identical.

Statistical analysis. Statistical significance was determined using the Least Significance Difference Test. All comparisons were two-sided and a *P* value of less than 0·05 was considered significant.

RESULTS

Mutants lacking *EFG1* have attenuated abilities to damage epithelial cells during infection of oral epithelium

The virulence phenotype after infection of RHE with the mutants *cph1*, *efg1* and *cph1*/*efg1*, the parental wild-type strain and the *EFG1* revertant was tested. After 12 h, epithelium inoculated with the parental strain, the mutants and the revertant was not significantly damaged. Thirty-six hours after infection, the *efg1* and the *cph1*/*efg1* mutants showed reduced epithelial damage (Fig. 1c, d) as compared

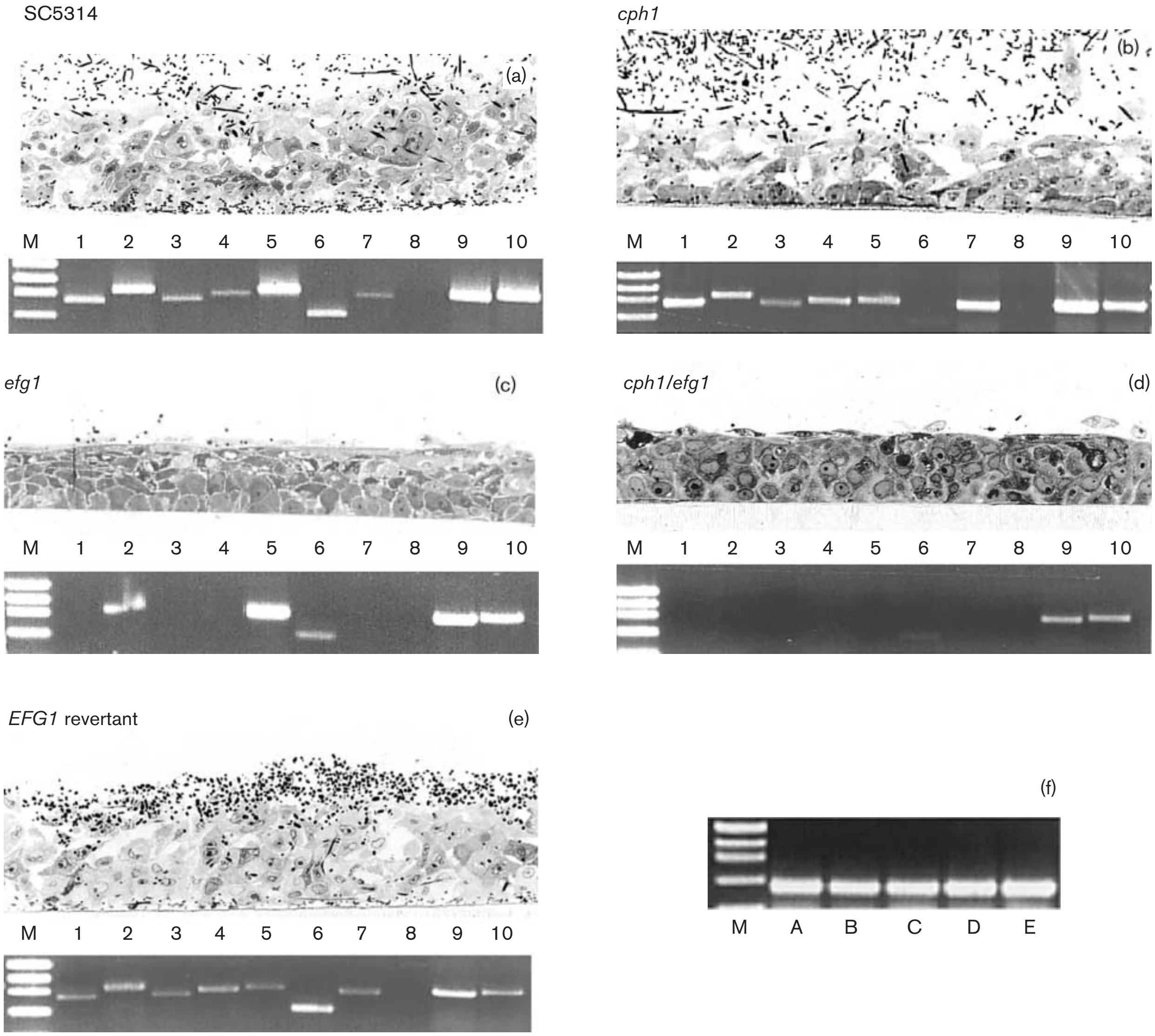

Fig. 1. Histological damage of RHE and *SAP1*–*10* expression for each of the *Candida* strains (lanes numbered 1–10) SC5314 (a), *cph1* (b), *efg1* (c), *cph1*/*efg1* (d) and the *EFG1* revertant (e) 36 h after infection. Vacuolization and oedema of all keratinocyte layers is accompanied by invasion of all epithelial layers by SC5314 (a), *cph1* (b) and the *EFG1* revertant (e). There was slight vacuolization and oedema of the superficial keratinocyte layers after infection of RHE with *efg1* (c) and *cph1*/*efg1* (d). Similar levels of cDNA and absence of contaminating genomic DNA in the samples are verified by equal amounts of *EFB1* transcripts and by a single PCR product of 564 bp (f).

with the wild-type (Fig. 1a) and the *cph1* infection (Fig. 1b). Plasmid-borne *EFG1* expression in the *efg1* mutant led to reconstitution of yeast-to-hyphal transition and epithelial lesions (Fig. 1e). These histological differences of the phenotype were confirmed by significant differences of LDH release as a marker of cell injury (Table 2).

Since the hyphal-deficient mutants *cph1*/*efg1* and *efg1* showed a strongly attenuated virulence phenotype we wondered if this is correlated with an altered *SAP* expression profile. Therefore, we studied *SAP* expression of these mutants by RT-PCR 12 and 36 h after epithelial infection and compared the expression pattern with those of the more virulent SC5314 wild-type parental, *cph1* mutant and *EFG1* revertant strains.

At 12 h, SC5314, *cph1* and *EFG1* revertant strains expressed transcripts of *SAP2*, *SAP5*, *SAP6*, *SAP9* and *SAP10* while for *cph1*/*efg1* and *efg1* only *SAP9* and *SAP10* cDNA was detected. These expression patterns were not accompanied by significant epithelial lesions.

Twenty-four hours later, SC5314 and *cph1* showed similar epithelial damage and a *SAP* expression pattern with transcripts for most *SAP* genes (Fig. 1a, b). In contrast, the reduced virulent phenotype of *efg1* and *cph1*/*efg1* mutants at the same time was accompanied by a lack of *SAP1*, *SAP3*, *SAP4* and *SAP7* expression (Fig. 1c, d). The revertant strain carrying a plasmid encoding the *EFG1* gene caused increased tissue damage and had the ability to express these *SAP* genes (Fig. 1e).

As seen in Fig. 1, the number of *C. albicans* cells was decreased in the samples which were infected with *EFG1*-deficient mutants. The majority of these cells were separated from the mucosal equivalent during the fixation and embedding process due to a reduced adherence. To rule out lower RNA levels isolated from these samples as the reason for the altered *SAP* expression profiles, we amplified the cDNA of the *C. albicans EFB1* gene (Maneu *et al.*, 1996), which is expressed in living *C. albicans* cells to a similar extent under all conditions and is therefore a useful internal standard (Schaller *et al.*, 1998, 1999). Similar expression levels of *EFB1* relative to the same amount of total RNA used in each RT-PCR experiment suggested that all strains were growing on the RHE tissue (Fig. 1f). A similar growth rate of all strains was confirmed by cell counting and/or microscopical observation of hyphal proliferation.

Table 2. Release of LDH by epithelial cells 36 h after infection with *C. albicans* parental and mutant strains

Data represent the analysis of three independent experiments (mean ± SD).

	LDH release (U l^{-1})
PBS	15 ± 6
SC5314	391 ± 15
cph1	372 ± 7
efg1	86* ± 7
cph1/*efg1*	44* ± 8
EFG1 revertant	224 ± 19

*Significantly different from values for SC5314, *cph1* and the *EFG1* revertant ($P < 0{\cdot}005$) as determined by the LSD test.

Mutants lacking *EFG1* are more susceptible to PMNs

To study the interaction of the hyphal-deficient mutants with host cells we investigated the resistance of the *cph1*, *efg1* and *cph1*/*efg1* mutants and the *EFG1* revertant to phagocytosis by PMNs in comparison with the parental strain (Fig. 2a–e). Normal morphology of *C. albicans* cells (Fig. 2f) and PMNs (Fig. 2g) after isolation of the cells was demonstrated by electron microscopy before the beginning of the experiments. Typical morphology of the PMNs was further proved after Giemsa staining with light microscopy to ensure that a pure population of neutrophils (>95 % purity) had been isolated. Vitality of the isolated PMNs without incubation with *C. albicans* was 98 % as monitored by the trypan blue staining method. At the ultrastructural level, 150 min after contact with PMNs, the great majority of wild-type, *cph1* mutant and *EFG1* revertant cells showed extensive hyphal formation and were localized extracellularly (Fig. 2a, b, e; Table 3). The PMNs that were in contact with these *C. albicans* cells often showed signs of necrosis (Fig. 2a, b, e; Table 3). In sharp contrast, most of the *efg1* and *cph1*/*efg1* cells were ingested by the PMNs (Fig. 2c, d; Table 3). Furthermore, escaping from the neutrophils by forming germ tubes was not observed. The host cells showed no alterations of the morphology while many of the engulfed yeast cells demonstrated signs of severe damage (Fig. 2c, d). Statistical analysis of the number of intra- and extracellularly located *C. albicans* cells and of damaged fungal and effector cells in the experiments clearly demonstrated an attenuated resistance of *cph1*/*efg1* and *efg1* mutants to neutrophil activities compared with the wild-type, *cph1* and the *EFG1* revertant (Table 3).

The viability of *C. albicans* parental, mutant and revertant strains was also compared by a killing assay. Cytotoxic effects of PMNs on *Candida* cells were analysed by a quantitative plate count method. After a defined period of exposure to PMNs, samples were plated on Sabouraud's agar. Controls included samples of these *Candida* strains without PMNs. After an incubation period of 24 h, growth of all strains was inhibited in the presence of PMNs. Similar inhibition rates were seen for the parental strain, the *cph1* mutant and the *EFG1* revertant while for *efg1* and *cph1*/*efg1* the number of survivors was significantly reduced (Table 3).

Lack of *SAP1* and *SAP3* expression by *cph1/efg1* and *efg1* mutants correlates with reduced levels of Sap1–3 protein

In former studies, we demonstrated the importance of Sap1–3 but not of Sap4–6 for virulence in experimental oral

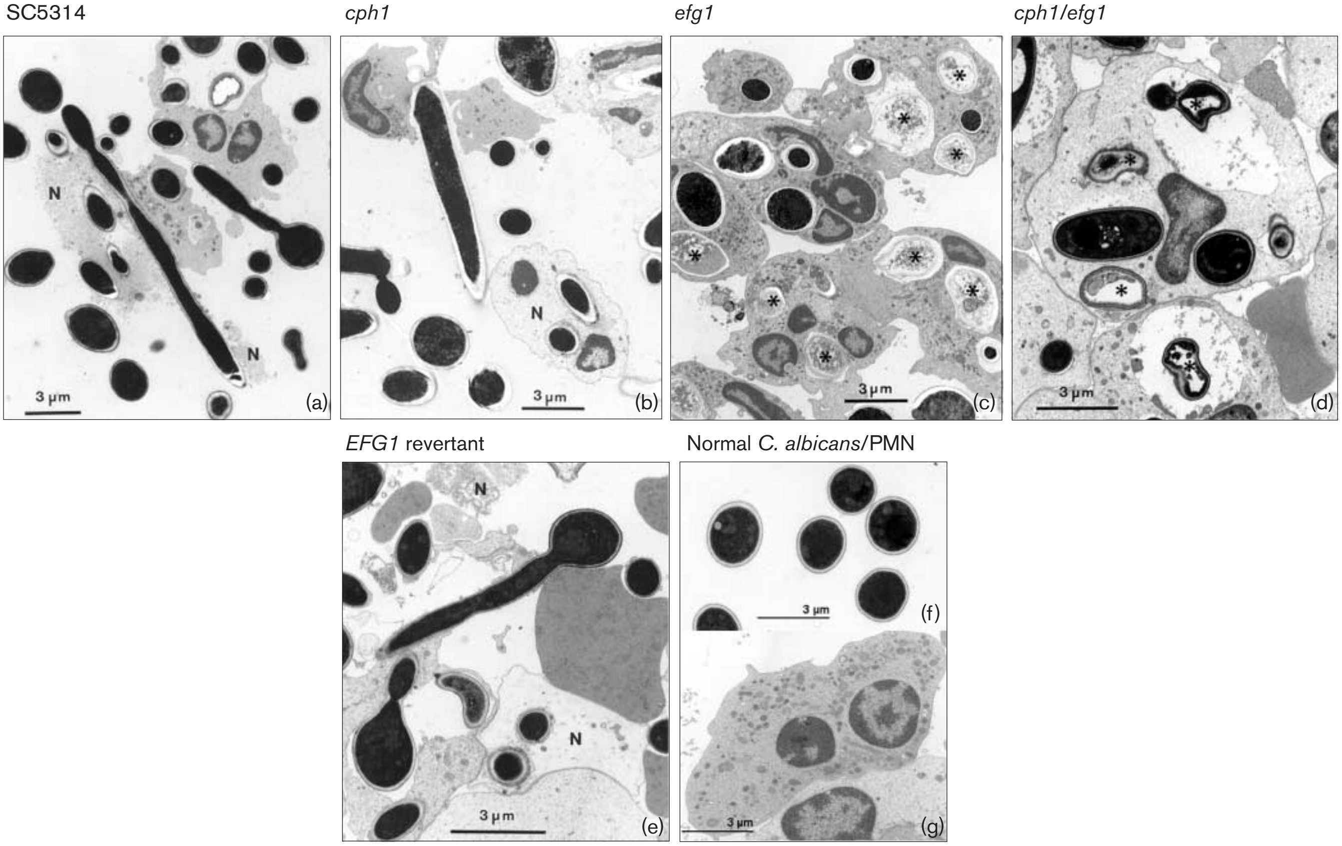

Fig. 2. Ultrastructural morphology 150 min after co-incubation of SC5314 (a), *cph1* (b), *efg1* (c), *cph1/efg1* (d) and the *EFG1* revertant (e) with PMNs, and normal morphology of undamaged *C. albicans* (f) and PMN (g) cells. The great majority of SC5314 (a), *cph1* (b) and *EFG1* revertant (e) cells are situated extracellularly and are regularly formed. In contrast, PMNs are highly damaged, demonstrating signs of necrosis (N) as oedema. *cph1/efg1* (d) and *efg1* (c) cells (*) are mainly intracellular and often highly damaged by healthy PMNs.

candidosis (Schaller *et al.*, 1999). We therefore focused our immunoelectron microscopical studies on Sap1–3 secretion during epithelial infection. As compared with the intensive anti-Sap1–3 labelling of each wild-type cell at 36 h the number of gold particles found within the great majority of the *cph1/efg1* and *efg1* mutant cell walls was strongly reduced. Plasmid-borne *EFG1* expression reconstituted an anti-Sap1–3 labelling of the *EFG1* revertant strain similar to wild-type cells (Fig. 3; Table 4).

Cytokine response is modulated only by the *cph1/efg1* double mutant

We investigated the *C. albicans*-induced expression of IL-1β, IL-8 and TNF of the RHE because a chemotactic signal for the recruitment of PMNs (IL-1β and IL-8) and a Th-1 response (TNF) is crucial for stimulating a protective immune response to the site of mucosal infection.

Non-infected RHE showed expression of genes encoding aldolase and GAPDH but no IL-1β, IL-8 and TNF transcripts. The wild-type and the *cph1* and the *efg1* mutants demonstrated a similar ability to stimulate epithelial expression of IL-1β, IL-8 and TNF 12, 36, 46 and 52 h after infection (Fig. 4). In contrast, the *cph1/efg1* mutant failed to stimulate epithelial IL-1β and IL-8 expression at 12 and 36 h, and TNF expression at 12, 36 and 46 h after inoculation of RHE (Fig. 4).

DISCUSSION

C. albicans possesses a variety of virulence attributes such as dimorphism, proteolytic enzymes and phenotypic switching necessary for tissue invasion (Calderone & Fonzi, 2001). In recent years it has been proposed that these single virulence factors may act synergistically to improve the virulence properties of the pathogen (Sonneborn *et al.*, 1999b; Schröppel *et al.*, 2000; Staib *et al.*, 2002; Felk *et al.*, 2002; Schweizer *et al.*, 2000; Srikantha *et al.*, 2000). Results from these experiments showed that transcription factors such as Efg1 or CaTec1 influenced the regulation of certain virulence attributes, for example phenotypic switching (Sonneborn *et al.*, 1999b; Srikantha *et al.*, 2000) or the expression of secreted

Table 3. Consequence of the interaction of *C. albicans* mutant and wild-type strains with PMNs

Percentage values represent the data after analysis of 30 representative electron microscopical figures (magnification ×5400) for each experiment (mean ± SD) and three independent killing assays.

Strain or genotype	*C. albicans* cells within PMNs		Damaged *C. albicans*		Damaged PMNs		Growth inhibition by PMNs (%)
	Mean	SD	Mean	SD	Mean	SD	
SC5314	4·5	1·8	0·5	0·7	2·1	1·7	28
cph1	4·6	1·5	0·5	0·8	2·8	1·2	19
efg1	9·3*	1·7	8·4*	1·7	0·7*	0·8	55*
cph1/efg1	12·9*	2·6	6·4*	1·4	0·2*	0·5	67*
EFG1 revertant	5·4	1·6	0·5	0·8	3·1	1·2	27

*Significantly different from values for SC5314, *cph1* and the *EFG1* revertant ($P < 0{\cdot}005$) as determined by the LSD test.

aspartyl proteinases (Schröppel *et al.*, 2000; Staib *et al.*, 2002; Felk *et al.*, 2002; Schweizer *et al.*, 2000). Investigations of a correlation between dimorphism and expression of proteinase genes in these studies concentrated on the hyphal-associated genes *SAP4–SAP6* (Schröppel *et al.*, 2000; Staib *et al.*, 2002; Felk *et al.*, 2002; Schweizer *et al.*, 2000). Recent gene array data have revealed that thousands of *C. albicans* genes are regulated during the yeast-to-hyphal transition, suggesting that a network of genes is involved during these morphological changes (Lane *et al.*, 2001). Our data demonstrate that the disruption of transcription factors which regulate the yeast-to-hyphal transition also has a consequence on the expression of hyphal-independent genes such as the proteinase genes *SAP1* and *SAP3*.

The majority of studies dealing with the virulence potential of *EFG1*- and/or *CPH1*-deficient mutants used murine or murine cell type models. Differences in the virulence of *cph1*, *efg1* single and the *cph1/efg1* double mutants were found in a mouse model for systemic infection (Lo *et al.*, 1997; Staib *et al.*, 2002; Felk *et al.*, 2002) and during interaction with mouse macrophages (Lo *et al.*, 1997). Investigations using human tissues were performed with endothelial (Phan *et al.*, 2000), intestinal and epidermal cells (Dieterich *et al.*, 2002). Results from these experiments demonstrated a significantly decreased virulence phenotype only in the mutants lacking functional *EFG1* but not for the *cph1* single mutant. In this study, we present evidence for an important role of dimorphism and hyphal-associated factors during invasion of human oral epithelium and during interaction with human neutrophils. These are novel contributions to the understanding of host–pathogen interactions in oral candidosis as oral epithelium is different from endothelial, epidermal or intestinal cells concerning morphology, function and expression of cytokines and markers of differentiation (Li *et al.*, 2000; Li & Thornhill, 2000; Uehara *et al.*, 2001). The interaction of murine macrophages with *CPH1*- and/or *EFG1*-deficient *C. albicans* mutants has been published by Lo *et al.* (1997). PMNs, however, are microphages and belong to the first line of host defence in oral candidosis (Eversole *et al.*, 1997; Challacombe, 1994; Farah *et al.*, 2001), while macrophages seem to play a more important role in deep-seated or systemic infections (Vazquez-Torres & Balish, 1997). Therefore, we decided to investigate the virulence phenotype of these mutants also during interaction with PMNs as an important host–fungus interaction in oral candidosis. Our results indicate that the Efg1-regulated PKA signal transduction pathway, but not the Cph1-regulated MAPK pathway, is important for the ability of *C. albicans* to damage human oral keratinocytes and to survive phagocytosis by human PMNs.

In previous studies, we have shown that *SAP1*, *SAP2* and *SAP3* are crucial for epithelial damage in a model of oral candidosis based on RHE (Schaller *et al.*, 1998, 1999). Therefore, we focused our studies on the expression of these *SAP* genes, which are especially important for this type of infection. In addition to morphological alterations, another possible reason for the attenuated pathogenicity of the hyphal-deficient *efg1* and *cph1/efg1* strains during infection of human oral epithelium might be a reduced level of *SAP* expression. During experimental oral infection, both virulence-attenuated mutants correspondingly failed to express detectable amounts of *SAP1* and *SAP3* transcripts as compared with the parental wild-type, the *cph1* mutant and the *EFG1* revertant strains. Decreased secretion of Sap1–3 by *efg1* and *cph1/efg1* was also confirmed on the protein level by immunoelectron microscopical studies. As shown in this study, deletion of *CPH1* did not reduce the ability to injure human keratinocytes and PMNs or change the *SAP* expression pattern during infection of human oral epithelium as compared with the parental strain. Modulation of *SAP* expression by *EFG1* was previously investigated by Schröppel *et al.* (2000) using Northern analysis. They demonstrated that *efg1* failed to express *SAP4*, *SAP5* and *SAP6* up to 4 h after induction under distinct *in vitro* hyphal-inducing condi-

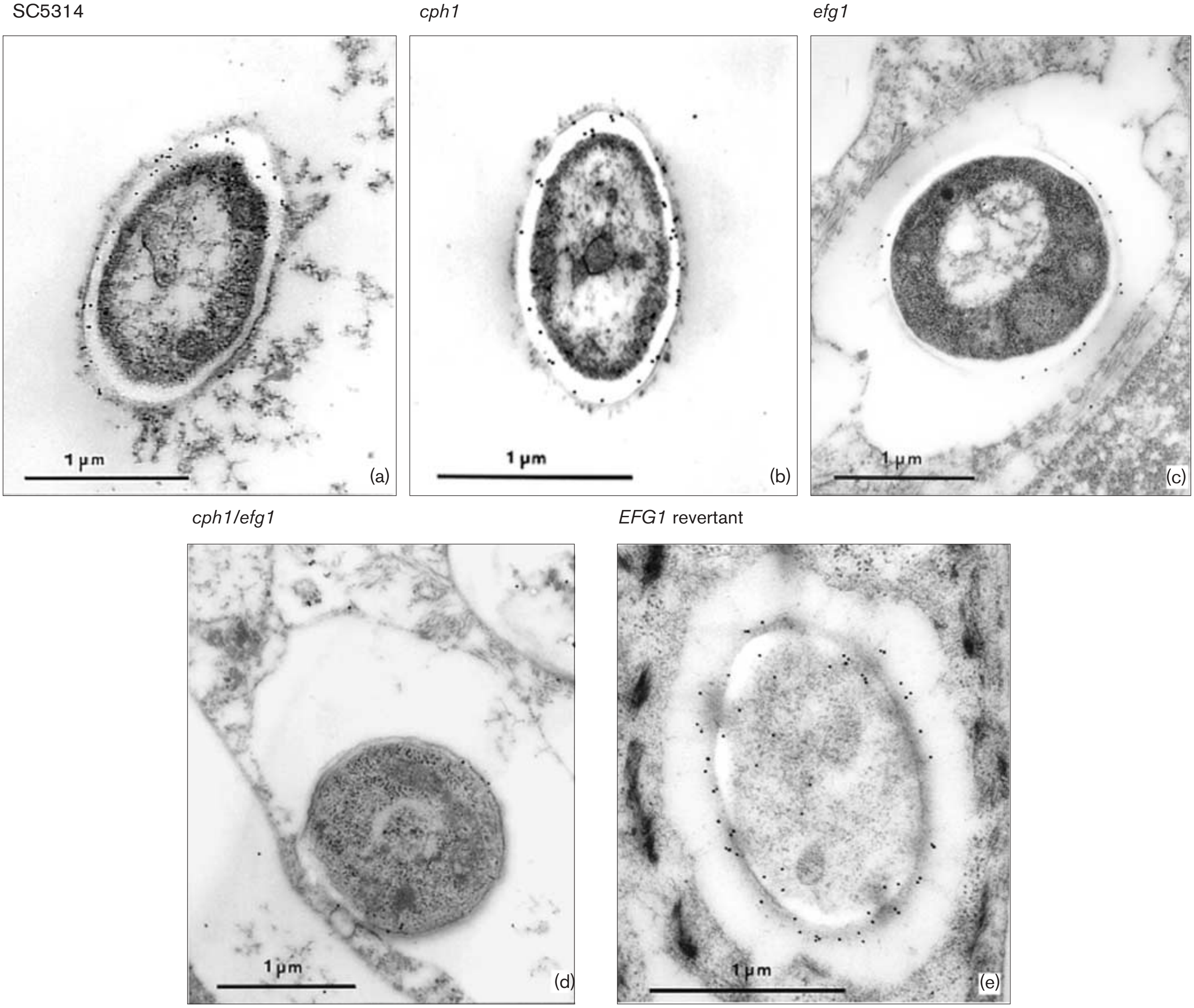

Fig. 3. Ultrastructural gold labelling of SC5314 (a), *cph1* (b), *efg1* (c), *cph1/efg1* (d) and the *EFG1* revertant (e) after incubation with antibodies directed against Sap1–3. There was strong gold labelling within the cell wall of SC5314 (a), *cph1* (b) and the *EFG1* revertant (e) cells. *efg1* (c) and the *cph1/efg1* (d) mutant cells are labelled with only a few gold particles.

tions. In our hands, using RT-PCR, expression of *SAP5* and *SAP6* by this mutant but not by *cph1/efg1* was detected during interaction with keratinocytes. A similar modulation of *SAP5* in the same mutants during systemic disease was found by Staib *et al.* (2002), who used an *in vivo* expression technology. In their study, transcript levels were reduced but not eliminated in *efg1* and completely lost in *cph1/efg1* during systemic mouse infection (Staib *et al.*, 2002). Felk *et al.* (2002) demonstrated strongly reduced transcript levels of *SAP4*, *SAP5* and *SAP6* by RT-PCR under certain, but not all, *in vitro* hyphal induction conditions and during *in vivo* expression in the peritoneal cavity of infected mice. These observed differences in these studies might be due to the various environmental conditions during the different growth conditions or types of infection, which may differentially modulate gene expression. Another possible reason might be the different sensitivity of the detection techniques used in each study. In addition to the well-known regulation of *SAP4*, *SAP5* and *SAP6* by *EFG1* we were able to demonstrate that expression of *SAP1* and *SAP3* is also modulated in mutants lacking this factor. It remains to be tested whether this is a direct consequence of a lack of transcriptional activation or an indirect effect, for example due to altered growth or virulence properties of these mutants. Amplification of the cDNA with primers for the house-keeping gene *EFB1* of *C. albicans* demonstrated similar expression levels for all investigated strains. Therefore, reduced cell growth of the mutants seems to be an unlikely explanation for the observed phenotypes.

Table 4. Levels of Sap1–3 secreted by *C. albicans* wild-type and mutants during oral candidosis (36 h)

Data represent the mean ± SD of gold particles found in 21 randomly selected cells.

Strain or genotype	Anti-Sap1–3 gold particles	
	Mean	SD
SC5314	61	11
cph1	55	13
efg1	15*	6
cph1/*efg1*	2*	2
EFG1 revertant	54	11

*Significantly different from values for SC5314, *cph1* and the *EFG1* revertant ($P < 0{\cdot}005$) as determined by the LSD test.

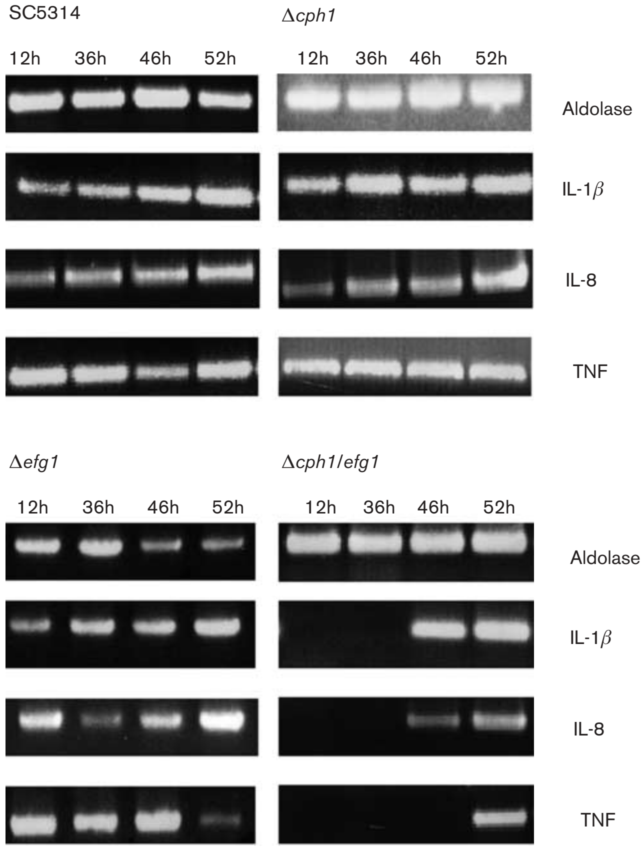

Fig. 4. Kinetics of cytokine expression (IL-1β, IL-8 and TNF) by RHE 12, 36, 46 and 52 h after stimulation with SC5314 (wild-type), *cph1*, *efg1* and *cph1*/*efg1*. Aldolase was used as an internal mRNA control. Results were obtained by RT-PCR. Failure of epithelial IL-1β and IL-8 expression at 12 and 36 h and TNF expression at 12, 36 and 46 h after infection of RHE with the *cph1*/*efg1* mutant as compared to cytokine expression after wild-type, *cph1* and *efg1* infection is shown.

Stimulation of cytokine and chemokine expression during infection of human oral epithelium was identical for the parental strain and the single mutants, while immune response was reduced for the *cph1*/*efg1* infection, suggesting a synergistic effect of the simultaneous deletion of *CPH1* and *EFG1*. Since the hyphal-deficient mutant *efg1* caused a similar cytokine expression pattern to wild-type cells, we concluded that hyphal morphology does not contribute significantly to the observed cytokine pattern. A similar pattern for expression of leukocyte adhesion molecules by endothelial cells induced by these different strains of *C. albicans* was demonstrated recently (Phan *et al.*, 2000).

Using a new set of highly specific *SAP1–10* primers, we were able to confirm our previous studies (Schaller *et al.*, 1998, 1999). We found a differential *SAP* gene expression pattern during experimental epithelial infection and could confirm that the presence of *SAP1* and *SAP3* transcripts correlates with the onset of epithelial lesions. Expression of *SAP2*, *SAP5* and *SAP6* was also seen in earlier stages without causing significant histological damage, indicating that these genes are not important for the development of a manifest oral disease as previously postulated by Naglik *et al.* (1999). In former studies, expression of *SAP9* and *SAP10* was not investigated. Here we show that both genes were expressed during all chosen growth conditions of the wild-type and the mutant strains, suggesting that the expression of these genes is independent from Cph1 and Efg1.

In summary, we present evidence that Efg1 is a key regulator for virulence during interaction with human epithelial cells and PMNs not only by regulating dimorphism but also by influencing the expression of hyphal-dependent and hyphal-independent aspartyl proteinase genes during experimental oral infection.

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