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State of differentiation defines buccal epithelial cell affinity for cross-linking to *Candida albicans* Hwp1

Gomathinayagam Ponniah[*], Christiane Rollenhagen, Yong-Sun Bahn[†], Janet F. Staab[‡], Paula Sundstrom

Department of Microbiology and Immunology, Dartmouth Medical School, Hanover, NH; and The Ohio State University, Columbus, OH, USA

Candida albicans **utilizes mammalian cell-associated transglutaminase (TGase) activity to adhere covalently to human buccal epithelial cells (BECs) through Hyphal Wall Protein 1. Little is known about the factors leading to the identity and appearance of Hwp1 binding partners on cells lining the oral cavity. The observation that BECs vary in their ability to attach to *C. albicans* germ tubes and to bind recombinant Hwp1 (rHwp1) suggested that differentiation may play a role in affinity for germ tube attachment. Individual BECs were characterized for differentiation status and rHwp1 binding. rHwp1 bound to the more terminally differentiated cells displaying SPR3 and keratin 13 but not to less differentiated cells with abundant involucrin. Sequential expression of involucrin followed by SPR3 in oral keratinocytes was demonstrated using stratified organotypic cultures and a feeder layer system with the OKF6/TERT-2 cell line. Increased cross-linking of the lysine analogue 5-(biotinamido)pentylamine to cultured OKF6/TERT-2 cell proteins accompanied this increased expression of SPR3. Western blot analysis demonstrated the presence of rHwp1 cross-links to proteins from BECs or from OKF6/TERT-2 cells that had been mechanically dislodged from culture dishes. Therefore, the differentiation of SPR3 positive from involucrin positive cells is correlated with the acquisition of affinity for cross-linking to rHwp1 and covalent adhesion of germ tubes to BECs.**

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Correspondence: Paula Sundstrom, Microbiology and Immunology HB3755, Dartmouth Medical School, Hanover, NH 03755, USA.
Tel: +1 603 650 1629; Fax: +1 603 676 1318, E-mail: paula.sundstrom@dartmouth.edu
[*]Present address: Department of Immunology, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA.
[†]Present address: Department of Bioinformatics and Life Science, Soongsil University, Seoul, Korea.
[‡]Present address: Oregon Health & Science University, Division of Infectious Diseases, Mail Code: NRC-03, 3181 SW Sam Jackson Park Rd., Portland, OR 97239, USA.
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Introduction

In clinical settings of immunodeficiency, disruption of the homeostatic mechanisms that prevent the excessive proliferation of the commensal, opportunistic pathogen *Candida albicans* commonly results in the development of white plaques that occur in oroesophageal candidiasis. Cells lining the oral cavity that are initially targeted for attachment by *C. albicans* compose the uppermost keratinocyte layer of the stratified squamous epithelium. Knowledge about the derivation of these cells from their progenitors as well as and their surface properties is essential for understanding the pathogenesis of candidiasis.

Keratinocytes lining the oral cavity that form the mucosal barrier are only temporarily positioned at the mucosal surface prior to exfoliation following proteolytic shaving of junctional proteins on their external perimeters. The surface is derived from basal keratinocytes through an ordered differentiation process that involves changes in gene expression that accompany changes in cell shape from plump with prominent nuclei in basal cells to flattened with pyknotic, fragmented or absent nuclei at the surface. This progression is also accompanied by changes in the expression of keratin isoforms from K19, expressed in the basal cells of non-keratinizing epithelia, to K4 and K13, which are expressed in differentiated cells of the buccal mucosa (1–3). Differentiation is classified into early and late stages by the expression level of proteins such as involucrin, an early, widely distributed differentiation marker in stratified squamous epithelial tissues (4) and SPR3, which is limited to oral and esophageal tissues (5, 6). Mature keratinocytes at the surface of the buccal stratified epithelium are characterized by the presence of cell-associated transglutaminase (TGase) activity present on both the external and internal faces of the cytoplasmic membrane (7). TGase

activity is essential for the formation of a specialized protective barrier through the cross-linking of epithelial-specific TGase substrates and the formation of a proteinaceous blanket of TGase-catalyzed isodipeptide bonds. Transgenic mice that lack epithelial TGase die within a few hours of birth (8).

Our efforts to understand the molecular mechanisms leading to oroesophageal candidiasis have led to the identification of a hypha-specific adhesin, Hwp1 (Hyphal Wall Protein 1), that functions as a substrate for TGases (9–11). Mutants with disruptions in the *HWP1* gene produce normal germ tubes but are deficient in stabilized adhesion to buccal epithelial cells (BECs) and are attenuated in murine models of candidiasis (12). The TGase substrate domain of Hwp1 is devoid of carbohydrate modification sites and is located at the N-terminus of the mature protein whereas the C-terminus undergoes modification with a glycosylphosphatidylinositol (GPI) anchor (13). The majority of Hwp1 molecules become cross-linked to the β-glucan of the cell wall through a GPI remnant; however, membrane anchored forms are also present. The central mucin-like domain of Hwp1 is heavily modified by carbohydrate addition through hydroxyl amino acids and asparagine linkages (14). Therefore, Hwp1 connects hyphae to BECs through covalent bonds at both ends of the protein.

Little data are available regarding the factors that permit the formation of a TGase-mediated protective barrier in epithelia that lack a cornified layer, such as the buccal epithelium studied in this report. It has been shown that internal 'wet' epithelia form an insoluble cross-linked protein layer beneath the cytoplasmic membrane of terminally differentiating keratinocytes that is approximately one-third the thickness of a similar layer found in epidermal keratinocytes undergoing keratinization (15) and that epithelial TGase, involucrin and other envelope precursor proteins are present and presumably function to construct protective barriers in non-keratinized regions of the oral cavity (3).

The relationships between the stage of oral keratinocyte terminal differentiation, level of TGase activity, adherence to *C. albicans* and affinity for Hwp1 cross-linking are unknown. In this work, individual oral keratinocytes were characterized in terms of their ability to adhere to *C. albicans* and form cross-links to recombinant Hwp1. The results suggest that the initiation of candidal lesions on the surface of stratified squamous epithelium depends on the presence of keratinocytes that are at a stage of differentiation in which TGase reactive co-substrates that can be cross-linked to rHwp1 are exposed on their cell surfaces.

Materials and methods

Preparation of BECs

Buccal epithelial cells were collected from buccal surfaces of informed, healthy volunteers who signed official consent forms that were approved by the human subjects committee at Dartmouth Medical School. Cells were collected by gently rubbing buccal surfaces with a sterile tongue depressor which was then swirled in 5 ml of sterile phosphate-buffered saline (PBS) (9 mM Na_2HPO_4, 2 mM $NaH_2PO_4{\cdot}H_2O$, 0.15 M NaCl pH 7.0). BECs were washed twice in PBS prior to use in indirect immunofluorescence or stabilized adhesion assays (9). Epithelial cells (SECs) from saliva (5 ml), processed as previously described (7, 16), were also collected from healthy volunteers. The stabilized adhesion assay was performed using non-radiolabeled wild-type *C. albicans* (SC5314) (17) germ tubes.

Production of rHwp1N13Vc-myc

To produce a recombinant TGase substrate domain of Hwp1 with a *c-myc* tag, the DNA region encoding Hwp1 residues 40–187 was amplified by the PCR with oligonucleotides 5′-GGG**ATCGAT**CGAAGCTCT TA-3′ and 5′-GCGC**TCTAGA**CCTGGAATGTTTGGAA TAGG-3′ with *Cla*I and *Xba*I sites (bold respectively) using pPICN13V as template. pPICN13V was derived from pPICN13 (13) following site-directed mutagenesis to create two threonine to valine changes in codons encoding amino acids 174 and 182 using the Gene Editor® *in vitro* site-directed mutagenesis system (Promega, Madison, WI, USA). The PCR product and the vector pPICZαC (3.6 kb) (Invitrogen, Carlsbad, CA, USA), encoding *c-myc* and 6X-his tags to engineer a fusion protein tagged at the C-terminus, were digested with *Cla*I and *Xba*I and incubated in the presence of T4 DNA ligase to generate pPICZαCrHwp1. The reading frame was found to be in frame with the *Saccharomyces cerevisiae* α-factor signal peptide by DNA sequencing. *Pichia pastoris* strain GS115 was transformed by electroporation with pPICZαCrHwp1 (5 μg) that had been linearized with *Sac*I. Expression and secretion of rHwp1N13V*c-myc* into the culture medium was performed as per the manufacturer's recommendations (Invitrogen). The culture medium containing rHwp1N13V*c-myc* was concentrated using Centriprep® (10 kDa) filter devices (Amicon; Millipore Corporation, Bedford, MA, USA), desalted using the Econo pac® 10DG disposable chromatography column (Bio-Rad Laboratories, Hercules, CA, USA) and purified by nickel column chromatography using Pro Bond® resin (Invitrogen) as per the manufacturer's instructions. Protein purity was determined by SDS-PAGE using conventional methods. TGase 2 substrate activity was assessed as previously described (13). rHwp1N13V*c-myc* formed cross-links with BEC proteins that were identical to those found with rHwp1N13 as assessed by SDS-PAGE and Western blotting (data not shown).

Determination of TGase activity in BECs and SECs

Paired BEC and SEC samples were collected on the same day from each of three healthy volunteers as described above. TGase enzyme activity was quantitated by comparison with purchased TGase (Sigma-Aldrich, St. Louis, MO, USA) prepared from guinea-pig liver with 2 units of activity (16). TGase lyophilized powder, reconstituted in 100 μl in double distilled water, was activated by the addition of 6.5 μl of 1 M DTT at room temperature (26 ± 2°C) and incubation for 8 h

(2 units/100 μl). Fluorescence of reactions containing from 0.01 to 0.04 U (0.5–2.0 μl) of activated TGase in a total volume of 500 μl of TGase reaction buffer (0.016 mM monodansylcadaverine, 100 mM Tris–HCl (pH 7.5), 5 mM $CaCl_2$, 2 mM EDTA, 1 mM DTT and 100 μg *N*,*N*-dimethylcasein (Sigma-Aldrich), were determined following a 15 min incubation at 37°C using a Wallac 1420 Victor2® fluorometer (Perkin-Elmer, Wellesley, MA, USA) with emission and excitation wavelengths of 500 and 350 nm respectively (18, 19). Fluorescence units of 2×10^5 BECs or SECs in 500 μl of reaction buffer was measured and the TGase activity was determined using a standard curve. Reactions without cells served as negative controls. Assays were performed in triplicate.

Indirect immunofluorescence assays on BECs

Detection of rHwp1N13Vc-myc:BEC binding

Buccal epithelial cells (1×10^6 cells) were washed in PBS and allowed to cross-link to 50 μg of rHwp1N13V*c-myc* in 300 μl of TGase reaction buffer. Reaction mixtures with 10 mM iodoacetamide or100 mM EGTA served as negative controls. The rHwp1 binding was detected with chicken anti-*c-myc* polyclonal (Aves Labs, Tigard, OR, USA) or mouse anti-*c-myc* polyclonal primary antibodies (Zymed, South San Francisco, CA, USA (1:20) and Alexa fluor 488 goat anti-chicken or goat anti-mouse secondary antibodies (1:50) (Molecular Probes, Eugene, OR, USA) by indirect immunofluorescence assays.

Simultaneous detection of rHwp1 cross-linking and EC proteins on BEC samples

Buccal epithelial cells that had been cross-linked to rHwpN13V*c-myc* were stained with anti-*c-myc* antibodies and with antibodies to one of the EC proteins using reagents described below.

Detection of TGase, involucrin, SPR3 and keratin 13

Transglutaminase was detected with mouse anti-human TGase monoclonal primary antibodies (Biomedical technologies, Stoughton, MA, USA) and Alexa fluor 488 goat anti-mouse secondary antibodies (Molecular Probes). Involucrin was detected with mouse anti-involucrin monoclonal primary antibodies (US Biological, Swampscott, MA, USA) and Texas Red goat anti-mouse secondary antibodies (Molecular Probes). SPR3 was detected with rabbit anti-SPR3 polyclonal primary antibodies [generously provided by C. Backendorf (6)] and Alexa fluor 488 goat anti-rabbit or Texas Red goat anti-rabbit secondary antibodies (Molecular Probes). Keratin 13 was detected with mouse anti-keratin 13 monoclonal primary antibodies (Sigma-Aldrich) and Texas Red goat anti-mouse secondary antibodies (Molecular Probes). In all cases, the dilutions of the primary and secondary antibodies were 1:20 and 1:50 respectively. Assays without primary antibodies or with ascites fluids (Mouse IgG1) (Chemicon, Temecula, CA, USA) served as controls. Images were acquired using an epi-fluorescence microscope (BX 60-Olympus, Center Valley, PA, USA) with a 20× or 40× objective equipped with a Magnafire® camera and Magnafire® 2.1 software (Optronics, Goleta, CA, USA). Fluorescent images collected with a single fluorochrome filter were superimposed on the corresponding DIC image whereas fluorescent images collected using two different fluorochrome filters were merged first and then superimposed on DIC image using Photoshop (ver. 6, Adobe, San Jose, CA, USA).

Quantitative assessment of involucrin and SPR3 levels on BECs from three donors

The level of antibody staining for simultaneous detection of involucrin and SPR3 on individual cells from three separate donors was graded according to the following scale: 4+, continuous bright opaque fluorescence over the entire cell so that the surface topology of the cell is not apparent; 3+, bright fluorescence in localized regions of the cell or continuous fluorescence over entire cell with the topology of the cell being discernible; 2+, intermediate level of fluorescence over the majority of the cell; 1+ faint but even fluorescence over the majority of the cell; ±, barely visible faint intermittent uncertain fluorescence; −, undetectable fluorescence.

Cell culture, fluorescence microscopy and transglutaminase assay

The oral mucosal cell line, OKF6/TERT-2 (20), a gift of J.G. Rheinwald, was initiated and expanded in keratinocyte serum-free medium (K-sfm; Invitrogen) at 37°C in 5% CO_2 in air atmosphere. The mouse fibroblasts, 3T3-Swiss albino CCL-92 (ATCC, Manassas, VA, USA) were cultured in 3T3 medium prepared from DMEM (Dulbecco's modified medium – pyruvate free) supplemented with L-glutamine (4 mM), glucose (4.5 g/l), sodium bicarbonate (1.5 g/l) and 10% bovine calf serum. After reaching 80–90% confluency, the cells were treated with 1 μg/ml mitomycin-C (Sigma-Aldrich) for 2.5 h in 3T3 medium and trypsinized with 0.05% trypsin-EDTA (Sigma-Aldrich) (21, 22). The mitomycin-C-treated 3T3 cells ($\sim 6 \times 10^6$ cells) were seeded onto cover slips, immersed in 10-cm plastic culture dishes, and cultured for 24 h in 3T3 medium. On the next day, 5.5×10^5 OKF6/TERT-2 cells per 10-cm dish were seeded onto the mitomycin-C-treated 3T3 cells and cultured in FAD medium (23, 24). The first cover slips were harvested on the next day (day 1). The concentration of calcium in the medium was increased stepwise from 0.6 to 0.9 mM $CaCl_2$ in 2-day intervals. Samples with cover slips were removed from the 10-cm dish at 24-h intervals, washed in PBS, and fixed with paraformaldehyde (PFA) solution (4% PFA, 4% sucrose in PBS) for 20 min. PFA was quenched with 50 mM ammonium chloride for 10 min, and cell membranes were permeabilized with 0.2% Triton X-100 for 5 min. Cover slips were washed twice for 5 min in PBS followed by blocking with 0.2% gelatin in PBS for 10 min. The primary antibodies, anti-SPR3 and anti-involucrin, were diluted 1:100 in PBS with 0.2% gelatin and applied for 1 h followed by two washing steps with PBS for 5 min. The secondary antibodies were diluted 1:100 in PBS with 0.2% gelatin, incubated on cells for 1 h, and washed twice with PBS.

Finally, cover slips were rinsed in distilled water and mounted with mounting medium (Sigma-Aldrich) on glass slides. Images were taken with a Zeiss fluorescence Axioplan2® microscope (Carl Zeiss, Inc., Thornwood, NY, USA) equipped with a Hamamatsu video C2400 camera and the HBO100 Mercury arc lamp as a light source. Images were processed with Metamorph® 4.6.5 software (Universal Imaging Corp., Downingtown, PA, USA). To assay cultured cells for the ability to form cross-links to exogenous substrates, OKF6/TERT-2 cells were cultured in the feeder layer system and the buccal cells were prepared as described above. On each day, cells in two 10-cm dishes were incubated with 4 mM 5-(biotinamido)pentylamine in culture medium for 3 h in the absence or presence of 1 mM cystamine. Two 10-cm dishes with 1×10^6 buccal cells each were treated similarly. Cells were washed with PBS, harvested with sample buffer, and loaded (30 μg per sample) onto a 7.5% SDS-PAGE gel. The gel was blotted onto a PVDF membrane, blocked with 5% milk TBST, and incubated with an HRP-avidin antibody in 5% milk/TBST for 1.5 h. ECL (GE Biosciences, Piscataway, NJ, USA) was used as a detection system.

Organotypic cultures

Organotypic cultures of OKF6/TERT-2 (20) were grown on a collagen matrix containing the human fibroblast cell line HFF-2 (ATCC). HFF-2 cells were grown in complete medium (DMEM with 4 mM L-glutamine, 4.5 g/l glucose, and 1.5 g/l sodium bicarbonate, 15% fetal bovine serum). OKF6/TERT-2 cells were expanded in keratinocyte-SFM medium (Invitrogen).

The organotypic culturing method was adapted from (25). Briefly, cultures were established in six-well tray and cover Sets with Transwells® (Organogenesis, Inc., Canton, MA, USA). One milliliter of acellular collagen matrix mixture was cast in each well and polymerized for 20 min, while a cellular fibroblast/collagen matrix was prepared by adding 3×10^5 HFF2 cells per ml to the collagen mixture. The fibroblast/collagen matrix (3 ml per well) was cast on top of the collagen matrix layer, polymerized for 30 min, and covered with fibroblast media by adding 10 ml to the bottom of each well and 2 ml on top of the collagen gel. Contraction of the collagen matrix occurred during incubation for 5–7 days at 37°C. A 50-μl suspension of TERT2 cells (3.5×10^6 cells/350 μl) in epidermalization media was pipetted onto the center of the collagen matrix of each well and allowed to sit undisturbed for 10 min to allow attachment of the cells to the matrix. This was followed by incubation at 37°C for another 30–60 min to ensure adhesion. Epidermalization media was carefully added to the top (2 ml) and bottom (10 ml) of the collagen matrix of each well. The tray was incubated at 37°C in a cell culture incubator and cells were fed every 2 days with epidermalization media. After 7 days, the culture was raised to an air–liquid interface by adding 6.5 ml of cornification media to the bottom part of each well. This culture was fed every second day for another 10 days.

Cryosectioning, immunostaining, and microscopy

The organotypic tissue was harvested by punching 8-mm circles from the tray where the organotypic culture was raised. The punches were washed with PBS and incubated in 2 M sucrose for 1 h at 4°C. Each punch was embedded with OCT compound (EMS, Hatfield, PA, USA) in a 2-ml test tube, snap frozen in dry ice, and stored at −80°C. Sections were performed by the department of research pathology at Dartmouth–Hitchcock Medical Center in Lebanon, NH. The sections were immunostained with the anti-SPR3 and anti-involucrin antibodies in a 1:1000 dilution. For detection, the Vectastain® universal ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used. The reagent 3-amino-9-ethylcarbazol (Vector Laboratories) was employed as a substrate for peroxidase. Sections were mounted with Fluoromount-G® (Southern Biotech, Birmingham, AL, USA) and visualized with an Olympus BX60 microscope as described above.

Epithelial TGase substrate activity of rHwp1

The assay was performed as previously reported (9, 13) except that the source of TGase was BECs (1×10^5 cells in 200 μl) prepared as described above and incubated with 20 μg rHwp1N13 (13) and 0.12 mM 5-biotinamidopentylamine in TGase reaction buffer for 1 h at 37°C. Iodoacetamide (15 mM) was added to the negative control reaction to inhibit TGase, and reaction was terminated by the addition of 100 mM EGTA (75 μl).

Detection of BEC and OKF6/TERT-2 protein:rHwp1 cross-links by Western blotting

Buccal epithelial cells (8×10^4 cells) were allowed to cross-link to 20 μg of rHwp1N13 (13) in TGase reaction buffer for 1 h. Iodoacetamide (1 mM) was added to the negative control reaction to inhibit TGase, and the reaction was terminated by the addition of 100 mM EGTA (75 μl). BECs were pelleted by centrifugation and washed in 500 μl of PBS. The supernatants were discarded and the pelleted cells were boiled in 50 μl of SDS sample buffer (26) containing β-mercaptoethanol, prior to separation of samples (2 μl) on a 12% SDS polyacrylamide gel. Samples were transferred onto Immobilon P® membranes (Millipore, Billerica, MA, USA) which were dried, blocked for 1 h in TBST containing 1% skim milk, washed twice for 5 min in TBST prior to inserting into a Decaprobe® apparatus (Hoefer, San Francisco, CA, USA) for multiple antibody detections and the addition of primary antibodies in TBST containing 1% bovine serum albumin for 1 h. Blots were washed four times for 5 min in TBST prior to the addition of secondary antibodies consisting of goat anti-rabbit or anti-mouse conjugated to HRP (New England Nuclear, Wellesley, MA, USA) at 37°C for 30 min. After four 10-min washes in TBST, membranes were developed using the ECL system (GE Biosciences). Molecular weights were estimated from a standard curve using Rainbow Molecular Weight Markers (Amersham Biosciences, Piscataway, NJ, USA) and Kaleidograph® software (Synergy Software, Reading, PA, USA) to create a standard curve. Primary antibodies used were

rabbit anti-rHwp1 diluted 1:1000 (10), rabbit antibodies to SPR3 diluted 1:2000, generously provided by C.M. Backendorf, mouse monoclonal antibodies to involucrin diluted 1:500, and keratin 13 diluted 1:2000 (Sigma-Aldrich). The results were repeated numerous times with multiple donors.

For the detection of rHwp1 cross-links in cultured cells, OKF6/TERT-2 cells grown for 6 days in feeder layer cultures and scraped from a 10-cm culture Petri dish (Fisher Scientific, Pittsburgh, PA, USA) were washed twice in PBS and incubated with 50 μg of rHwp1N13V*c-myc* 50(g) for 1 h under TGase reaction conditions. The reaction was stopped by the addition of 100 mM EGTA and 10 mM iodo-acetamide. Cells were washed three times with PBS prior to the addition of SDS sample buffer (500 μl) and boiled for 5 min. The extract (13 μl) was loaded into each lane and the cross-linked products were detected by SDS-PAGE and Western blotting using antibodies to *c-myc* (1:1000).

Results

The possibility that extracellular changes associated with keratinocyte maturation might lead to the surface expression of epithelial TGase co-substrates important for Hwp1 binding was suggested by the variability seen among BECs from the same donor in the number of attached *C. albicans* germ tubes using the standard stabilized adhesion assay (9) (Fig. 1A–C). Variability among BECs in the cross-linking of recombinant Hwp1 (rHwp1N13V*c-myc*) under transglutmaminase reaction conditions was also observed (Fig. 1D), suggesting that differences exist among individual BECs in the availability of specific co-substrates for rHwp1 cross-linking as well as for overall germ tube adherence. Minimal rHwp1 was associated with BECs when TGase activity was inhibited (Fig. 1E).

Assessment of the presence of differentiation markers involucrin, SPR3, and keratin 13 on individual BECs

To investigate whether BECs at different stages of maturation were present in each donor sample, we first analyzed cells simultaneously for the presence of an early (involucrin) and a late (SPR3) differentiation marker by indirect immunofluorescence (Fig. 2A). Cells exhibiting strong staining for both markers were not found, which is consistent with the simultaneous presence of cells in different stages of differentiation. In addition, the majority of BECs were positive when stained with antibodies to TGase (data not shown).

To further characterize the presence of SPR3 and involucrin on individual BECs in terms of relative levels of expression, over 300 cells from each of three separate donors were assessed using the criteria described in Materials and methods (Table 1). Overall, SPR3 was the predominant marker. Over 10% of cells in all donors were singly positive for SPR3, whereas involucrin-positive, SPR3-negative cells were rare in donors 2 and 3. Of the cells that were positive for both markers, SPR3 was present in higher levels than involucrin [2+ to 4+ for SPR3 vs. 1+ for involucrin (19.8%, 16.8%, and 34.0%)], than vice versa [2+ to 4+ for involcrin vs. 1+ for SPR3 (5.1%, 4.5%, and 0%)] for all donors. High levels (3+ to 4+) of expression of both proteins in the same cell were not found (Table 1 and Fig. 2A). For all donors, the largest proportion of doubly positive cells

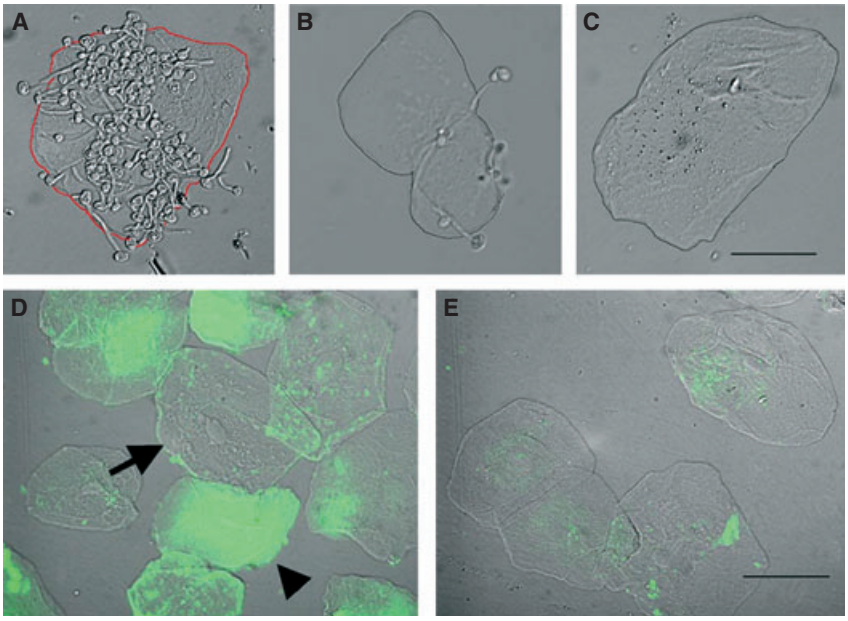

Figure 1 Variable *Candida albicans* adherence and TGase mediated cross-linking of rHwp1 to buccal epithelial cells from the same donor. (A–C) Examples of BECs with many (A), few (B), or no (C) attached germ tubes following the stabilized adhesion assay using *C. albicans* strain SC5314 (9). (D–E) Detection of rHwp1 on BECs after incubation in TGase cross-linking conditions using indirect immunofluorescence. (D) Individual BECs showed strong (arrow head) or weak (arrow) cross-linking with rHwp1 as detected with anti-*c-myc* antibodies. (E) Negative control reactions included iodoacetamide to inhibit TGase. Scale bar is 50 μm.

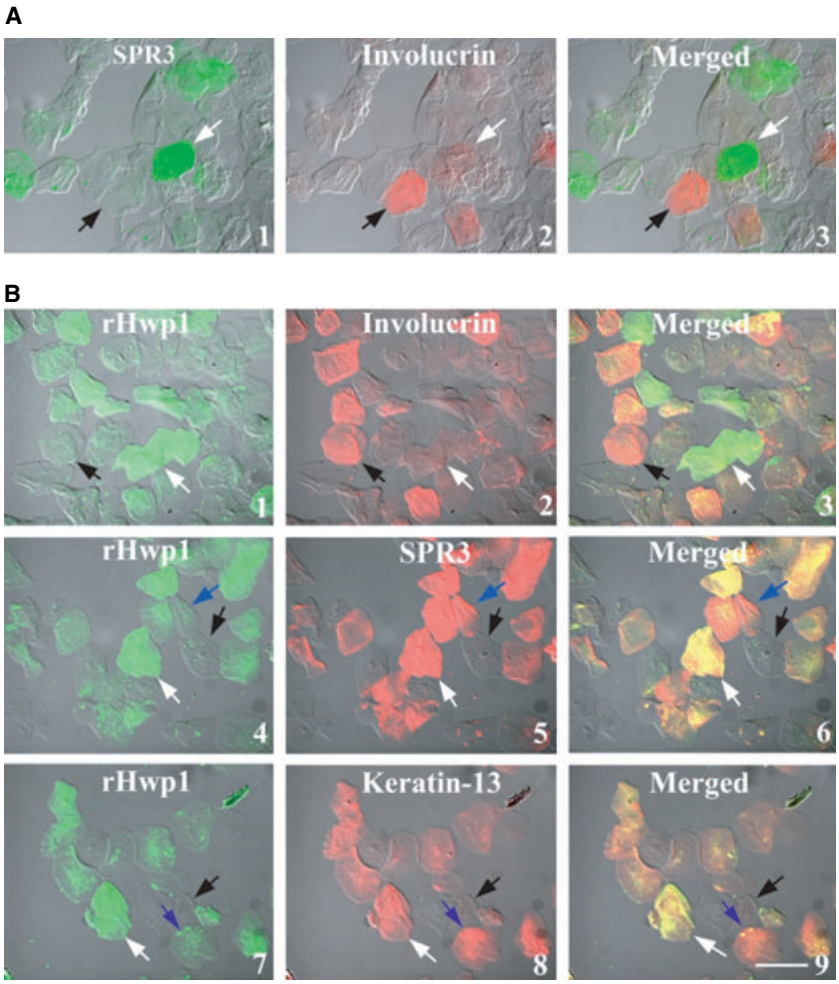

Figure 2 Pattern of involucrin, SPR3 and keratin 13 expression, and rHwp1 cross-linking to BECs. (A). Presence of cells at different stages of differentiation in BEC populations. Indirect immunofluorescence detection of SPR3 (1) and involucrin (2) on BECs collected from the same donor. Cells with abundant involucrin (black arrows) were weakly positive or negative for SPR3. Cells with abundant SPR3 (white arrows) were weakly positive or negative for involucrin. Scale bar is 50 μm. (B). Correlation of rHwp1 binding with presence of SPR3 and keratin 13 but not involucrin. BECs that bound rHwp1 (white arrows), stained weakly with antibodies to involucrin (2–3) but stained strongly with antibodies to SPR3 (5–6), or keratin 13 (8–9). Many cells that were not susceptible to rHwp1 binding (black arrows) had undetectable levels of SPR3 (5–6) or keratin13 (8–9) but were strongly positive for involucrin (2–3). BECs that stained strongly with antibodies to SPR3 or keratin 13 but did not bind rHwp1 were also detected (4–9, blue arrows). Scale bar is 50 μm.

exhibited weak (1+) staining for both markers (33.3%, 41.1%, and 32.4%). The sampling technique used by each donor may have contributed to the observed donor variation; however, the differences between donors were consistent.

Correlation of epithelial differentiation markers and rHwp1N13Vc-myc binding

To determine whether rHwp1 bound to BECs with either early or late markers or with both classes of markers, cells were incubated with rHwp1 under TGase cross-linking reaction conditions. Antibodies to *c-myc* as well as involucrin, SPR3, or the terminal differentiation marker keratin 13 (2, 3, 27, 28) were used for the simultaneous detection of rHwp1 and epithelial cell proteins. rHwp1 binding was found to be directly correlated with the presence of SPR3 and keratin 13 and inversely correlated with the presence of involucrin (Fig. 2B). However, the presence of SPR3 and keratin 13 were not absolute predictors of rHwp1 cross-linking capacity on BECs as cells with abundant SPR3 or keratin 13 and low levels of rHwp1 binding were also found. Also, the merged images did not show a perfect overlap between rHwp1 and SPR3 or keratin 13.

Ordered expression of involucrin and SPR3 on differentiating immortalized oral keratinocytes

To study the relative timing of SPR3 and involucrin expression, immortalized OKF6/TERT-2 keratinocytes derived from the floor of the mouth were differentiated on fibroblast feeder layers in increasing concentrations of calcium (Fig. 3). Involucrin was initially actively synthesized in FAD medium on day 1 as previously described (20), and high levels were maintained on day 2 upon increasing the calcium concentration of the FAD medium from 0.3 to 0.6 mM. The prominent increase in

Table 1 BuccalCell Expression of Involucrin and SPR3[a,b,c] (A) Levels of involucrin or SPR3 expression on cells displaying a single marker[d] (B) Levels of involucrin and SPR3 expression on cells displaying both markers[d]

SPR3	*Donor 1,2,3*	*Involucrin*	*Donor 1,2,3*
A			
+ + + +	0, 0, 0[e]	+ + + +	0, 0, 0
+ + +	1.4, 1.0, 1.7	+ + +	1.9, 0, 0
+ +	3.8, 4.5, 6.1	+ +	8.4, 1.5, 0
+	7.5, 5.0, 10.6	+	16.4, 2.0, 0.6

	SPR3			
Involucrin	+ + + +	+ + +	+ +	+ +
B				
+ + + +	0, 0, 0[e]	0, 0, 0	0	0.9, 0, 0
+ + +	0, 0, 0	0, 0, 0	0.9, 2.0, 0	1.4, 1.0, 0
+ +	0, 5.9, 5	1.4, 8.9, 3.9	0, 6.9, 5.6	2.8, 3.5, 0
+	1.4, 0.5, 2.2	8.5, 6.4, 10.6	9.9, 9.9, 21.2	33, 41.1, 32.4

[a]The levels of SPR3 and involucrin were evaluated following indirect immunofluorescence according to the criteria described in the Materials and methods section.
[b]The total number of cells evaluated for donor 1, 2 and 3 was 371, 379 and 344, respectively.
[c]The number of cells that were positive for one or both markers for donor 1, 2 and 3 was 213, 202, and 183 respectively.
[d]Percentages relative to the total number of positive cells for each donor.
[e]Donor 1, Donor 2, Donor 3.

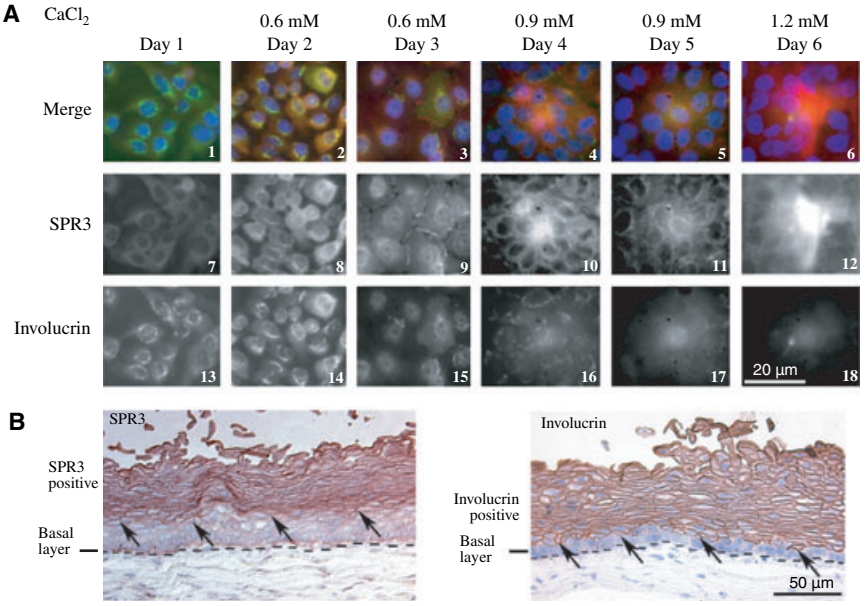

Figure 3 Timing of involucrin and SPR3 expression in the OKF6/TERT-2 oral keratinocyte cell line following differentiation in the presence of calcium. (A) Indirect immunofluorescence of OKF6/TERT-2 cells permeabilized with Triton X-100 and grown on fibroblast feeder cells at the indicated Ca^{2+} concentrations to stimulate differentiation. Involucrin is shown in green and SPR3 in red on the merged images (1–6). Note the increase in SPR3 expression on day 2 in image 8 compared with day 1 in image 7 and the sustained high level of expression through day 6 (9–12). The yellow color on the merged image on day 2 indicates an abundance of both markers. Involucrin appears decreased on day 3 in image 15 compared with day 2 in image 14 and remains at this low level through day 6 (images 16–18). (B). Immunostains of OKF6/TERT-2 organotypic cultures grown above a collagen matrix. Reactivity with antibodies against SPR3 and involucrin is indicated by a red color, whereas nuclei are stained blue with hematoxylin. The interface between the basal layer of keratinocytes and the collagen matrix is shown by a dotted line. Involucrin antibodies were detected in every cell layer except for the basal layer (arrows). The basal layer and three to four cell layers above the basal layer were weakly reactive with SPR3 antibodies, whereas higher layers were strongly positive.

SPR3 synthesis observed on day 2 showed that SPR3 synthesis followed involucrin synthesis. The most differentiated cells on days 3 and 4 continued to display a high level of SPR3 synthesis with SPR3 becoming more evenly spread throughout the cytoplasm on day 3 prior to becoming concentrated at the cell peripheries on day 4. Meanwhile, the decreased detection of involucrin on days 3 and 4 was probably the result of loss of exposure

of reactive epitopes due to the incorporation of other proteins into the cross-linked scaffold as involucrin epitopes were not found to be decreased in differentiated cells in superficial layers of organotypic cultures (see below). Nevertheless, the results clearly showed an upregulation of SPR3 during differentiation.

Ordered expression of involucrin and SPR3 in differentiating organotypic cultures of immortalized oral keratinocytes

As previously described, involucrin was observed in all cell layers above the basal layer in cultured keratinocytes (29). By contrast, SPR3 was weakly detectable in the basal cells and in the three to four layers of cells closest to the basal cells. SPR3 was prominently increased in higher cell layers, consistent with the expression of SPR3 in more differentiated cells when compared with involucrin. These results are consistent with the sequential increase in the expression of involucrin and SPR3 observed in the feeder layer cultures. However, a decline in involucrin expression was not observed in the upper layers of the organotypic cultures. This result suggested that involucrin epitopes were exposed in cross sections of organotypic cultures and that the observed decrease in involucrin in the feeder layer system was related to the reduced accessibility of antibody to involucrin epitopes.

Overall, the culture results are consistent with the assertion that cells that stain strongly with SPR3 in BEC samples are more mature than other cells. The most mature cells exhibit SPR3 at the surface, whereas less developed cells are weakly positive for both SPR3 and involucrin.

Cross-links between rHwp1 and BEC or OKF6/TERT-2 proteins

In a previous work, rHwp1 was shown to be a substrate for TGase2 using [^{14}C]putrescine as a co-substrate (9). To demonstrate that rHwp1 is also a substrate for BEC TGase activity, BECs were incubated with rHwp1 and the soluble co-substrate 5-biotinamido-pentylamine (5BPA) under TGase reaction conditions (Fig. 4A). 5BPA was found to conjugate to rHwp1 as detected with streptavidin-HRP, suggesting that rHwp1 indeed serves as a substrate for BEC TGases.

The presence of complexes between BEC proteins and rHwp1 was assessed following incubation with rHwp1 under TGase cross-linking conditions by an analysis of BEC proteins released after boiling in SDS sample buffer. For these experiments, an untagged version of rHwp1 was used (see Materials and methods); however, similar results were found with the tagged version as well. Cross-linked bands would be expected to migrate more slowly than monomeric rHwp1 and to bind rHwp1 antibodies. Whereas monomeric rHwp1 migrated at 33 kDa, a discrete band of approximately 93 kDa as well as polydisperse material near the top of the gel were detected in crude extracts from BECs that had been incubated in the presence of rHwp1 under TGase reaction conditions but not when TGase was inhibited with iodoacetamide (Fig. 4B). These cross-linked species were not recognized by antibodies to involucrin, SPR3 or keratin 13, indicating that SPR3 and keratin 13 do not become cross-linked to rHwp1 but rather serve as markers of the differentiation state in which cells are susceptible to rHwp1 cross-linking (data not shown).

Given that the OKF6/TERT-2 cells cultured on fibroblast feeder layers showed an ordered expression of differentiation markers, it was of interest to determine whether a concurrent TGase-mediated activity capable of cross-linking extracellular substrates to epithelial proteins could be demonstrated. The lysine analogue 5BPA was added to OKF6/TERT-2 cells during growth in culture and the presence of biotinylated peptides was assessed using streptavidin conjugated to horse radish peroxidase as previously described (13, 30). Over time, increasing amounts of two streptavidin-reactive bands of 126 and 162 kDa in size were detected that were not found in control reactions which included cystamine to inhibit TGase activity (Fig. 4C). These results showed that OKF6/TERT-2 cells undergoing differentiation expressed increasing amounts of extracellular cross-linking activity. TGase-mediated cross-linking activity was also found to be associated with BECs, although the cross-linked bands were more heterogeneous in size.

In contrast to the results with 5BPA, OKF6/TERT-2 cells in culture were not able to cross-link rHwp1 (data not shown). However, if the OKF6/TERT-2 cells were mechanically removed from the culture dish prior to incubation with rHwp1, a 93-kDa cross-linked band and material that barely entered the gel could then be detected in OKF6/TERT-2 keratinocytes after 6 days of culture in the feeder layer system (Fig. 4D). Although the reasons for the differing results using rHwp1 vs. 5BPA in unperturbed OKF6/TERT-2 cell cultures are unknown, one possibility is that the small size of 5BPA (MW 328.5 Da) allowed for its accessibility to OKF6/TERT-2 co-substrates. In summary, the ability of cells to cross-link extracellular substrates is acquired during the process of differentiation. However, the accessibility of exogenous substrates to OKF6/TERT-2 TGase cross-linking activity may vary depending on the properties of the substrate.

Discussion

Oroesophageal candidiasis involves attachment to and invasion of specialized epithelial lining cells that have matured following numerous changes in their gene expression, cytoskeletal arrangements, and cornified envelope assembly that markedly distinguish these mature cells from their basal keratinocyte precursors. The potential importance of TGase activity associated with these cells for candidiasis was recognized after the discovery of the *C. albicans* TGase substrate Hwp1 (9). However, details regarding the molecular mechanisms of attachment of *C. albicans* Hwp1 to keratinocytes, particularly with regard to the processes involved in the generation and exposure of epithelial co-substrates serving as binding partners for Hwp1, are unknown. The mechanisms leading to cross-linking by Hwp1 are

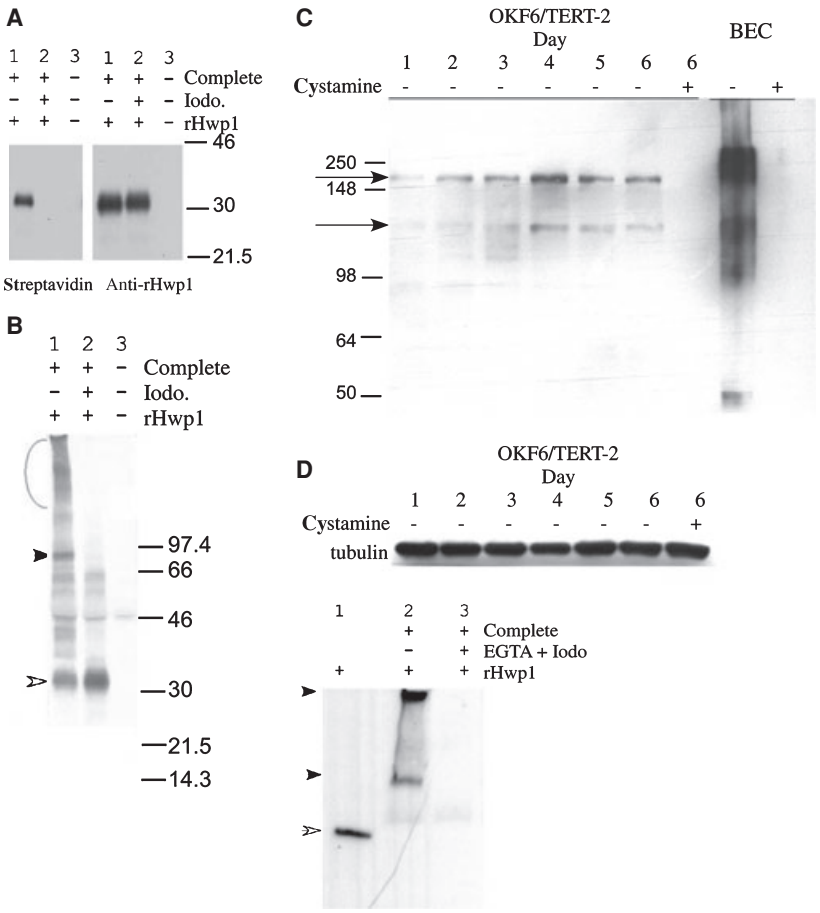

Figure 4 Cross-linking of rHwp1 to BEC and OKF6/TERT-2 proteins. (A) rHwp1N13 was incubated by itself with 5-biotinopentylamine (5BPA) and BECs as a source of TGase under TGase reaction conditions. Reaction products were detected with streptavidin conjugated to horseradish peroxidase at a dilution of 1:2000 (Zymed Laboratories, Inc.) (left panel) or anti-Hwp1 (right panel). 5BPA was cross-linked to rHwp1 in the complete reaction (left panel, lane 1, arrow) but not in the presence of TGase inhibitors (left panel lane 2). Reaction products were not detected in the absence of rHwp1 (lane 3). Reactions inhibited with iodoacetamide contained amounts of rHwp1 equivalent to the complete reaction (right panel, compare lanes 1 and 2). (B). BECs were incubated with rHwp1N13 under TGase cross-linking conditions prior to extraction in SDS sample buffer, SDS-PAGE, and Western blotting. A 93-kDa band (black arrow) and polydisperse bands at the top of the gel (brackets) representing rHwp1N13 cross-linked to unknown proteins were found in the complete reaction (lane 1) but not in control reactions with iodoacetamide (lane 2). The bands between monomeric rHwp1 and the 97-kDa marker represent aggregates of rHwp1 that were present in the absence of BECs (data not shown). A minor background band was detected in BECs in the absence of rHwp1N13 (13) (lane 3). (C). Detection of cross-links between 5BPA and OKF6/TERT-2 proteins. Extracts prepared from OKF6/TERT-2 cells that had been incubated with 5BPA in transglutaminase (TGase) reaction conditions contained proteins of 126 and 162 kDa (arrows) that were detected with streptavidin conjugated to horseradish peroxidase following SDS-PAGE and blotting. The 162-kDa proteins reached maximal levels on day 4. Cross-linked bands were not detected in control reactions that included cystamine to inhibit TGase. Detection of tubulin showed that equivalent amounts of samples were loaded in each lane. (D). Detection of cross-links between rHwp1 and OKF6/TERT-2 proteins. OKF6/TERT-2 cells that had been mechanically removed from culture dishes were incubated with rHwp1N13V*c-myc* under TGase cross-linking conditions prior to extraction in SDS sample buffer, SDS-PAGE, and Western blotting using antibodies to *c-myc*. Cross-linked rHwp1 species consisting of a 93-kDa band and high molecular weight material at the top of the gel (solid arrows) were detected with antibody to *c-myc*. The first lane contains 10 ng of purified rHwp1N13V*c-myc*. The position of monomeric rHwp1 proteins is indicated by open arrowheads (B and D).

potentially important for the onset of candidiasis in multiple host sites as well as for colonization because of the tropism of *C. albicans* for stratified squamous epithelium.

The suggestion that cellular differentiation status played a role in the generation of Hwp1 anchoring sites came with the discovery that rHwp1 binding occurred with BECs harboring high levels of SPR3 and keratin 13 but not involucrin. The finding that the three donors whose BECs were analyzed all showed a predominance of the SPR3 marker in BEC populations compared with involucrin suggested that the presence of BECs with SPR3 and, thus, their affinity for rHwp1 attachment, is a general property of buccal keratinocytes.

Although SPR3 and keratin 13 were correlated with rHwp1 binding, they were not themselves co-substrates for Hwp1 in TGase-mediated cross-linking. The absence of uniform co-localization between rHwp1 and SPR3 or keratin 13, as shown in overlays of the two markers on BECs, combined with the presence of BECs with high levels of SPR3 or keratin 13 that did not bind rHwp1, argues against a direct role for SPR3 or keratin 13 in the formation of cross-links to rHwp1. The 93-kDa band and polydisperse bands, which were much higher in

molecular weight than un-cross-linked Hwp1 (Fig. 4D), were not recognized by antibodies to SPR3 and keratin 13 or by antibodies to SPR1 or SPR2 (data not shown), suggesting that the stage at which rHwp1 binds to BECs occurs within a window of time in which SPR3 and keratin 13 are both prominent.

The profiles of involucrin and SPR3 expression on BECs from three donors, in combination with the results from differentiating keratinocyte cultures, suggested that keratinocytes lining buccal surfaces may be in the process of transitioning from the expression of involucrin to the expression of SPR3. The ordered expression of involucrin followed by SPR3 in OKF6/TERT-2 cells that accompanied increased Ca^{2+} levels is consistent with the hypothesis that involucrin-positive BECs are the forerunners of SPR3-positive BECs. Importantly, the ability to cross-link exogenous TGase substrates was also correlated with differentiation, as shown by the increasing levels of proteins in OKF6/TERT-2 cells that became cross-linked to 5BPA over time. Maximal levels were seen on day 4, in which SPR3 was mostly detectable at the perimeters of the cells as shown by indirect immunofluorescence.

The detection of rHwp1 cross-linking to proteins in OKF6/TERT-2 cells required that the cells be mechanically removed from the culture dish. This result suggested that the observed cross-linking capacity of BECs might also be dependent on the mechanical forces that are employed in sample collection, implicating a necessity for exposing either TGase or BEC TGase substrates for the covalent cross-linking of Hwp1 to BEC proteins. The mechanisms leading to the exposure of TGase substrates *in vivo* are unknown. However, it is possible that the penetration of germ tubes in to the BEC layer lining the mucosa, or other factors such as dehydration or mild abrasion, could expose such sites in hosts that are predisposed to candidiasis.

Keratinocyte differentiation status suggests a partial explanation for the observed variability in rHwp1 cross-linking and germ tube adhesion shown in the Results section. High levels of rHwp1 cross-linking are predicted to involve BECs with high levels of SPR3 and keratin 13 that also express an undefined keratinocyte TGase co-substrate that cross-links rHwp1, whereas cells that bind less rHwp1 may be in the process of differentiating and are not yet mature enough to express high levels of SPR3 and the keratinocyte co-substrate. Alternatively, cells that do not bind rHwp1 may have been subjected to proteolytic events associated with exfoliation that removed the cross-linking determinants.

The findings from this study are consistent with previous results pointing to the importance of TGase(s) in rHwp1 cross-linking in interactions with keratinocytes. Inhibition of TGase activity abrogated rHwp1 binding to BECs and prevented formation of a 93-kDa complex representing rHwp1 epithelial protein cross-links in BEC preparations and in OKF6/TERT-2 cells. Although the specific TGases acting on rHwp1 in candidiasis are unknown, TGase 1, which is localized to the cytoplasmic membrane during differentiation, is the most likely TGase to be involved in interactions with extracellular substrates. TGase 2 and TGase 3 have also been shown to act on rHwp1 *in vitro* (9), and there is no reason to expect that rHwp1 would not serve as a substrate for other TGases.

The possibility that low levels of germ tube adhesion and rHwp1 binding to some BECs was related to low levels of TGase activity is unlikely. TGase was found on nearly all of the BECs by indirect immunofluorescence (data not shown), consistent with its known expression in all layers of human epidermis, with a 100-fold upregulation in mRNA and protein in terminally differentiating keratinocytes (31, 32), and with earlier reports showing that BECs have TGase activity (7). Furthermore, naturally exfoliated keratinocytes in saliva were found to have higher levels of TGase activity than BECs, yet keratinocytes in saliva do not attach to *C. albicans* germ tubes (data not shown). The ubiquitous presence of TGase in the context of variable rHwp1 cross-linking emphasizes the importance of an appropriate state of differentiation to facilitate covalent interactions of *C. albicans* with BECs.

One of the benefits of this study is the development of a model of the buccal epithelium. Given the patterns of differentiation marker exposure in human BEC populations, it appears that the organotypic OKF6/TERT-2 culture system produces stratified layers of cells which may be manipulated to generate surfaces that emulate those of the human mucosa. We know of no studies that examined the distribution of both involucrin and/or SPR3 in normal oral mucosa using immunohistochemical techniques applied to frozen sections or formalin-fixed tissue. Thus the relative timing of the appearance of involucrin and SPR3 in stratified layers of normal tissue is not known. SPR3 is expressed in the stratum spinosum of buccal tissue (33) and involucrin is expressed above basal cell layers (34). Our analysis of human BECs argues against identical kinetics for expression of these two markers and the analysis of OKF6/TERT-2 cells differentiated on feeder cells strongly supports the sequential expression of involucrin followed by SPR3. This model will be useful for studying oral epithelial interactions with *C. albicans* in the context of the normal differentiation status and the relevant anchoring mechanisms that lead to invasion. One limitation of the OKF6/TERT-2 culture system is that elements of the immune response are not present. Immunity undoubtedly influences invasion of the epithelium and may also influence initial germ tube attachment in the host although such immune factors do not interfere with the cross-linking capacities of BECs collected from human donors. Our data support the utility of the model for separating out interactions that are specific to differentiated epithelial cells that may form the initial interactions of *C. albicans* with BECs. The model may also prove useful in future research for studying the impact of elements of the immune system on interactions of BECs with germ tubes.

The results presented in this work are the first to address the importance of epithelial differentiation at the molecular level in the context of *C. albicans*

adherence and invasion. These results are essential for the identification of pharmaceutical agents that will inhibit candidal invasion of epithelial lining cells through interference with TGase-mediated attachment of rHwp1 to epithelial co-substrates generated during the process of differentiation and cornified envelope assembly. In addition, the findings have a direct bearing on understanding the complexities of stimulation and paralysis of host antifungal defenses, such as defensin production and the chemoattraction of phagocytic cells in the context of differentiation. Finally, the results suggest that the mechanisms that serve to keep the concentration of *C. albicans* cells in oral secretions low or absent, as well as host factors that prevent the formation of *C. albicans* Hwp1-coated germ tubes, are important in suppressing overgrowth of *C. albicans* by minimizing interactions with keratinocytes exhibiting differentiation characteristics that lead to their affinity for rHwp1 cross-linking and germ tube attachment.

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