Identification of salivary basic proline-rich proteins as receptors for Candida albicans adhesion

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INTRODUCTION

The yeast Candida albicans forms part of the normal human oral microbial flora and can be isolated from the mouths of 20–40% of healthy individuals (Odds, 1988). Under conditions of host immunosuppression, C. albicans can cause a variety of mucosal infections, including pseudomembranous candidosis, erythematous candidosis, hyperplastic candidosis, Candida-associated denture stomatitis and Candida-associated angular cheilitis (Odds, 1988; Scully et al., 1994). Indeed, oral candidosis is a prognostic indicator for AIDS, with 33–50% of HIV-seropositive and AIDS patients developing oral candidosis, which can lead to serious oesophageal infections (Samaranayake & Holmstrom, 1989; Samaranayake, 1992).

Oral surfaces are coated with salivary pellicles formed by the selective adsorption of components from whole saliva and from other minor secretions, e.g. serum exudate (Sonju et al., 1974). Pellicle maturation involves additional intermolecular protein interactions and enzymatic modification, such as the covalent linking of proline-rich proteins (PRPs) to buccal epithelial cells (Bradway et al., 1992). Proteinaceous pellicles provide a range of immobilized receptors for microbial adhesion (Jensen et al., 1992). An initial step in the establishment of oral candidosis involves the adhesion of yeast cells to saliva-coated host tissues and so it is important to study the interaction between Candida cells and components of salivary pellicles. An understanding of the structure and function of the molecules involved in this interaction may enable the development of methods to prevent the adherence of Candida, or promote the clearance of Candida cells from the oral cavity.

C. albicans cells bind to a number of receptors, including fibronectin (Skerl et al., 1984) and the H sugar sequence found on all blood group substances of the ABO[H] system (Brassart et al., 1991). Adherence of C. albicans...
cells to a single component of human whole saliva, provisionally identified as low-molecular-mass mucin MG2, and to rat submandibular gland (RSMG) mucin (Hoffman & Haidaris, 1993) has also been demonstrated. However, it was later shown that purified mucins did not promote the adhesion of C. albicans to polymethylmethacrylate (Edgerton et al., 1993) and that the C. albicans-binding component of RSMG was in fact a proteoglycan (Hoffman & Haidaris, 1994). Similarly, we have previously demonstrated that parotid saliva fractions containing mucins did not promote the adherence of C. albicans cells to hydroxyapatite beads (Cannon et al., 1995). C. albicans cells were found to adhere to fractions of saliva containing PRPs, which have also been shown to promote adhesion of Streptococcus gordonii to apatitic surfaces (Gibbons et al., 1991). The objective of this study was to further characterize components from parotid saliva that act as receptors for C. albicans adherence.

**METHODS**

**Yeast strains, media and culture conditions.** The following C. albicans strains were used in this study: ATCC 10261; I1 (hp11an; Schmid et al., 1990); I17 (hp50an; Schmid et al., 1995); I33 (HMHC4; Schmid et al., 1990); and I44 (sw-17c; Schmid et al., 1995). C. albicans cells were grown in glucose salts biotin medium (GSB; Holmes & Shepherd, 1988) at 30 °C with shaking (200 r.p.m.). Radiolabelled C. albicans cells were prepared by inoculating GSB (40 ml), containing 2.9 mM pepstatin, 4 °C. The apparent molecular mass of proteins was estimated according to the method of Laemmli (1970) at 15 V cm⁻¹ and 16 °C. The specific activity (c.p.m.)⁻¹ with a mean value of 19 ± 5.2 cells (c.p.m.)⁻¹ for 18 h at 4 °C with reciprocal shaking (70 min⁻¹). Membranes were washed four times in PBS/0.1% Tween 20 to remove nonspecifically bound C. albicans cells and air-dried prior to exposure to X-ray film.

**Protein analyses.** Solid-phase protein samples on PVDF membranes were N-terminally microsequenced by automated Edman degradation (Hubbard, 1995). Protease digestion was performed by the method of Cleveland et al. (1977). Proteins were separated on 16% polyacrylamide gels, stained with Coomassie blue, and portions of the gel containing specific bands were excised. These proteins were digested with 0.06-0.6 units endoproteinase Glu-C (sequencing grade; Promega). Digestion was performed within a polyacrylamide stacking gel, with no current for periods of 10 or 20 min, and the products were then separated by electrophoresis through a 16% polyacrylamide gel (25 V cm⁻¹, 22 °C).

Proteins were prepared for mass spectrometry (MS) by extraction from nitrocellulose with trifluoroacetic acid (TFA) and acetonitrile using the method of Harrington (1990). Samples were freeze-dried and resuspended in 5–10 μl 0.1% TFA, to a concentration of 5–10 pmol μl⁻¹. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was performed using a Finnigan Lasermat 2000 MALDI-TOF mass analyser. The mass spectrometer was calibrated against the α-cyanohydroxycinnamic acid matrix peak at 3794 Da.

**RESULTS**

**Binding of C. albicans cells to electrochemically separated parotid saliva proteins**

Human parotid salivary proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes and incubated with radiolabelled Candida cells. C. albicans ATCC 10261 cells bound reproducibly to 16 proteins with molecular masses of between 17 and 63 kDa. There were a number of immobilized protein bands which did not stain intensely with Ponceau S but...
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Fig. 1. Comparison of C. albicans ATCC 10261 cell adherence to electrophoretically separated parotid salivary proteins with that of clinical strains 11, 117, 133 and 144. Parotid salivary proteins were separated on a 12.5% polyacrylamide gel and electroblotted onto nitrocellulose membranes. The membranes were incubated with 30 ml radiolabelled cells (1.2–1.5 × 10^7 cells ml^-1). Experiments were performed at least three times. Representative results are shown. (a) Parotid saliva (15 pg) stained with Ponceau S. (b) Autoradiogram of parotid saliva Western blots (15 pg protein per lane) incubated with C. albicans ATCC 10261 (lane 1), C. albicans 11 (lane 2), C. albicans 144 (lane 3), C. albicans 117 (lane 4) and C. albicans 133 (lane 5). Arrowheads indicate the subset of proteins to which all Candida strains tested bound.

Conversely, several protein bands that stained intensely with Ponceau S did not act as receptors for C. albicans ATCC 10261 cell adherence. A subset of four parotid salivary protein bands with molecular masses of 17 (band A), 20 (band B), 24 (band C) and 27 (band D) kDa (Fig. 1) promoted the adherence of C. albicans ATCC 10261 cells, even at low concentrations of parotid salivary protein (2 µg per lane). The binding of various C. albicans clinical isolates to parotid salivary proteins was compared. There was strain-specific variation in the proteins to which cells adhered and in the proportion of input cells adhering to certain protein bands (Fig. 1). However, all strains tested bound to bands A–D (Fig. 1), and so these proteins were investigated further. Comparisons of the molecular masses of proteins A–D with the published migration positions of the major human parotid salivary proteins (Beeley et al., 1991) indicated that they may be basic PRPs (bPRPs; molecular masses of 17 and 20 kDa), acidic PRP A (27 kDa) and an unspecified PRP (24 kDa).

In the hydroxylapatite-bead adhesion assay, a greater proportion of clinical isolate I1 cells bound to saliva-coated hydroxylapatite beads than did ATCC 10261 cells (Table 1). The proportion of strain I44 cells binding to beads was slightly less than that for ATCC 10261 and the proportion of adherent cells was markedly decreased for strains I17 and I33. Interestingly, the amount of cell binding to proteins on the overlay assay (Fig. 1) correlated with the adherence measured using the saliva-coated hydroxylapatite-bead adhesion assay. All strains were labelled with [35S]methionine to the same specific radioactivity.

Table 1. Adherence of C. albicans strains to saliva-coated hydroxylapatite beads

<table>
<thead>
<tr>
<th>C. albicans strain</th>
<th>10^{-3} × No. of cells adhering (± SD)</th>
<th>Adherence relative to C. albicans ATCC 10261 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 10261</td>
<td>3.66 ± 0.59</td>
<td>100</td>
</tr>
<tr>
<td>I1</td>
<td>5.07 ± 0.42</td>
<td>112.5 ± 9</td>
</tr>
<tr>
<td>I17</td>
<td>1.95 ± 0.42</td>
<td>53.4 ± 1.15</td>
</tr>
<tr>
<td>I33</td>
<td>0.53 ± 0.07</td>
<td>13.8 ± 1.6</td>
</tr>
<tr>
<td>I44</td>
<td>2.34 ± 0.38</td>
<td>87.5 ± 13.8</td>
</tr>
</tbody>
</table>

Fractionation of parotid saliva

Parotid saliva was fractionated by column chromatography (Fig. 2) using a modification of the method of Strömberg et al. (1992). Fractions containing protein peaks, judged by the A_{226}, were pooled (Fig. 2). The
proteins present in these fraction pools were concentrated by dialysis against NH$_4$HCO$_3$ and lyophilized and resuspended in KCl buffer. The protein-banding pattern of the pooled parotid salivary proteins after SDS-PAGE and Ponceau S staining was not affected by concentrating the pools. Pools 1–4 each contained several proteins with a wide range of molecular masses (Fig. 3). This may indicate that under the nondenaturing chromatography conditions, proteins formed heterocomplexes which were subsequently resolved into component proteins by SDS-PAGE. Comparisons of the molecular masses of the electrophoretically separated proteins suggested that pools 1–4 contained proline-rich glycoproteins (PRGs) and PRPs (Stromberg et al., 1992). Hydroxylapatite-bead adhesion assays confirmed that pools 1–4 promoted the highest levels of C. albicans adherence. Blot overlay assays carried out on the fraction pools indicated that most of the binding of C. albicans cells occurred to proteins present in pools 2, 3 and 4 (Fig. 3). The C. albicans cells bound to the same subset of proteins identified in overlay assays using unfractionated saliva (Fig. 1). Pools 6–12 contained very few proteins and gave a similar banding pattern in the overlay assay to pool 5, except that there was no binding to bands A or B (data not shown).

**Characterization of bands A, B, C and D**

Bands A and B were electrophoretically separated from pool 3 and bands C and D were separated from parotid saliva. They were then transferred to PVDF membrane (Towbin et al., 1979) and excised. Approximately 180 pmol band A and band B was sequenced, and each was found to have the same N-terminal amino acid sequence, NH$_3$-SPPGKPGQGPPQGNNQPGQG, with no indication of a contaminating sequence. Bands C and D had the same N-terminal sequence, NH$_3$-SPPGKPGQG. A comparison of the N-terminal sequence of bands A and B with protein databases resulted in five matches with 100% similarity over the 20 amino acids (Table 2). All matches were with human parotid salivary non-glycosylated bPRPs. As these proteins (IB-6, IB-7, IB-8a and IB-9) each have the same N-terminal sequence (Kauffman et al., 1991), identification of the proteins in bands A, B, C and D beyond being members of the non-glycosylated bPRP family could not be made at the level of N-terminal amino acid sequence.

Band A was chosen for further analysis as it was the smallest member of the parotid salivary protein subset to which C. albicans cells bound. Endoproteinase Glu-C (V8 proteinase) cleaves proteins on the carboxyl side of glutamic acid or aspartic acid residues. The predicted endoproteinase Glu-C digestion products for IB-6, IB-7, IB-8a and IB-9 are given in Table 2. Endoproteinase Glu-C digestion of band A produced a single

**Table 2. Proteins with the same N-terminal amino acid sequence as bands A and B**

<table>
<thead>
<tr>
<th>Protein identity</th>
<th>Predicted molecular mass* (Da)</th>
<th>Predicted endoproteinase Glu-C digestion products (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB-6 (Kauffman et al., 1986)</td>
<td>11518</td>
<td>7274; 4262</td>
</tr>
<tr>
<td>IB-7 (Kauffman et al., 1991)</td>
<td>5770</td>
<td>5285; 503</td>
</tr>
<tr>
<td>IB-8a (Kauffman et al., 1991)</td>
<td>12430</td>
<td>5285; 5285; 1886</td>
</tr>
<tr>
<td>IB-8c (Kauffman et al., 1991)</td>
<td>5843</td>
<td>5843</td>
</tr>
<tr>
<td>IB-9 (Kauffman et al., 1982)</td>
<td>6025</td>
<td>5313; 730</td>
</tr>
</tbody>
</table>

* Molecular mass was calculated using the GeneJockey program (Biosoft; Cambridge).
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Fig. 4. Endoproteinase Glu-C digestion product of band A. Band A was subjected to endoproteinase Glu-C digestion and then electrophoresis through a 16% polyacrylamide gel. The gel was stained with Coomassie blue by the method of Cleveland et al. (1977). Lanes: 1, low-molecular-mass markers; 2, band A; 3, band A plus 0.6 units endoproteinase Glu-C; 4, band A plus 0.3 units endoproteinase Glu-C; 5, band A plus 0.06 units endoproteinase Glu-C; 6, 0.6 units endoproteinase Glu-C. A, Band A; Ap, endoproteinase Glu-C digestion product of band A.

product band (Ap) detected by Coomassie blue staining (Fig. 4). Ap had an apparent molecular mass of 7.4 kDa, compared with an apparent molecular mass of 15.2 kDa for band A. Amino acid sequencing of protein band Ap indicated that there were two predominant peptides present with N-terminal sequences SPPG and GNPP. The sequence of one peptide (designated ApN), SPPG, corresponds to the N-terminal sequence of band A and other bPRPs, while the sequence of the other peptide (designated ApC), GNPP, corresponds to that which might be expected after endoproteinase Glu-C cleavage of bPRP IB-6 or IB-8a. However, endoproteinase Glu-C cleavage of IB-8a should result in three, rather than two, fragments (Table 2). The endoproteinase Glu-C digestion product (Ap) of band A did not act as a receptor for C. albicans adhesion in overlay assays (Fig. 5).

MS of band Ap detected two species of masses 6189.07 ± 602 and 4261.14 ± 277 Da (mean ± SD of seven determinations). The mass of one of the species, 4261 Da, matched that expected for the C-terminal fragment of IB-6 (Table 2). The mass of the other species was 1085 Da smaller than that expected for the N-terminal endoproteinase Glu-C fragment of IB-6, and did not correspond to any of the cleavage products predicted for IB-6, IB-7, IB-8a, IB-8c or IB-9. The mass of the protein comprising band A could not be determined by MS.

DISCUSSION

Saliva is a complex mixture of ions, proteins, glycoproteins and polysaccharides of host and microbial origin (Gibson & Beeley, 1994). A number of salivary proteins and glycoproteins are adsorbed onto oral surfaces to form the acquired pellicle and in so doing provide many potential receptors for microbial adhesins (Bradway et al., 1992). The objective of this study was to identify receptors in human parotid saliva for the adhesion of C. albicans.

Parotid saliva was studied because it can be obtained relatively free of microbial contaminants, and it was pooled in order to remove the effects of individual-to-individual saliva variations (Beeley et al., 1991). The overlay assay has been used in a number of attempts to identify possible salivary receptors for microbial adhesion (Prakobphol et al., 1987; Gillece-Castro et al., 1991; Murray et al., 1992; Hoffman & Haidaris, 1993, 1994). Binding of Fusobacterium nucleatum to the low-

Fig. 5. Binding of C. albicans ATCC 10261 cells to band A and the endoproteinase Glu-C digestion product, Ap. Proteins were separated by electrophoresis through a 16% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. (a) Ponceau-S-stained nitrocellulose membrane. Lanes: 1, low-molecular-mass markers; 2, parotid saliva (15 μg); 3, fractionated parotid saliva pool 3 (7.5 μg); 4, band A; 5, band A plus 0.6 units endoproteinase Glu-C; 6, band A plus 0.3 units endoproteinase Glu-C. (b) Autoradiogram of the membrane in (a) probed with C. albicans ATCC 10261 cells. Lanes are the same as in (a). A, Band A; Ap, endoproteinase Glu-C digestion product of band A.
and high-molecular-mass PRGs present in human submandibular-sublingual saliva (Prakobphol et al., 1987) has been demonstrated. Later work showed that F. nucleatum binds to the low- and high-molecular-mass PRG, via interactions with the unsubstituted lactosamine units, including the 6-antennae of the major oligosaccharide residues (Gillece-Castro et al., 1991).

Murray et al. (1992) studied the binding of 16 strains of streptococci to salivary glycoproteins present in parotid and submandibular-sublingual saliva. Three strains (Streptococcus sanguis 72-40 and 804, and Streptococcus sobrinus OMZ176) bound to single components, two to mucin MG2 and one to an unidentified protein, and five strains (S. sanguis 10556, S. gordonii 10558 and G9B, and Streptococcus oralis 10557 and 72-41) bound to multiple components, including MG2, α-amylase and PRGs.

Our previous study indicated that C. albicans bound to proteins in five separate pools of fractionated parotid saliva when adsorbed to hydroxylapatite beads (Cannon et al., 1995). Therefore, it was not surprising that radiolabelled C. albicans cells bound to several electrophoretically separated parotid saliva proteins, immobilized on nitrocellulose in the overlay assay. We have also found that C. albicans cells bound to a similar set of proteins from unstimulated human whole saliva in overlay assays (unpublished observations).

Strain-specific variation was evident in the overlay profiles; however, a subset of four proteins acted as receptors for all C. albicans strains tested. The fact that C. albicans bound to several parotid saliva proteins may indicate the presence of either common receptors on different parotid salivary proteins, or multiple adhesins on the surface of C. albicans. We observed that strain-specific variation in the proportion of input cells binding to parotid saliva proteins in the overlay assay correlated with the level of adhesion observed in saliva-coated nitrocellulose-adhesive assays. These variations may be related to either the avidity of adherence, or the number or accessibility of adhesins on the cell surface.

C. albicans cells bound to at least four proteins with sequence homology to nonglycosylated bPRPs (bands A–D). Protease digestion of band A with endoproteinase Glu-C resulted in a single band detected by Ponceau S or Coomassie blue staining after SDS-PAGE. This band contained two protein species with molecular masses of 4261 and 6189 Da, and two predominant N-terminal amino acid sequences. The presence in the digestion product of peptides with an N-terminal amino acid sequence and a molecular mass predicted for the C-terminal product of endoproteinase Glu-C digestion of bPRP IB-6 indicates that the protein in band A may be bPRP IB-6. The N-terminal fragment appears to be a degradation product of the N-terminal endoproteinase Glu-C fragment of IB-6 which is co-migrating with the smaller fragment. Discrepancies between the apparent molecular mass of band A and the calculated molecular mass are explained by the observation that PRPs migrate anomalously in SDS-PAGE (Anderson et al., 1982). It appears that endoproteinase Glu-C cleavage of band A results in loss of the C. albicans adhesion property. This may be due to proteolytic destruction of the adherence epitope, or conformational changes as a result of proteolysis.

The binding of C. albicans cells to immobilized bPRPs suggests that these salivary proteins may have a role in the adhesion of Candida cells in the oral cavity. C. albicans ATCC 10261 cells have been shown to adhere to both a carbohydrate moiety and proteins on the surface of immobilized S. gordonii cells (Holmes et al., 1995b, 1996). Pretreatment of immobilized S. gordonii NTCC 7869 cells with whole saliva enhanced C. albicans ATCC 10261 cell binding (Holmes et al., 1995a). Since S. gordonii NTCC 7869 and other primary colonizers of the enamel pellicle such as S. parasanguis and S. sanguis adsorb bPRPs (Heaney et al., 1992; Newman et al., 1993), it may be that these micro-organisms become coated with bPRPs and provide receptors for the binding of C. albicans. We are investigating whether C. albicans cells bind PRPs in the fluid phase. It has been shown that oral bacteria bind to adsorbed PRPs despite the presence of fluid-phase PRPs (Gibbons et al., 1991), and this may also be the case with C. albicans. Preliminary assays have shown that preincubation of C. albicans cells with parotid saliva did not detectably reduce the levels of PRPs.

Johnsson et al. (1993) predict that IB-6 may be capable of interacting with hydroxylapatite and thus form part of the enamel pellicle. However, mucosal surfaces are the major sites of oral colonization by C. albicans (Arendorf & Walker, 1980). The enzyme transglutaminase has been shown to covalently link acidic PRPs to epithelial cells and may also be involved in the attachment of bPRPs to mucosal surfaces (Bradway & Levine, 1993). Therefore, it is possible that transglutaminase-catalysed linkage of bPRPs to epithelial cells may be another factor promoting the adherence of C. albicans cells. Alternatively, bPRPs adsorbed to bacteria already attached to epithelial cells may present adhesion receptors for C. albicans colonization.

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