THE ROLE OF SALIVARY AGGLUTININ AND STATHERIN IN THE DEFENCE AGAINST MICRO-ORGANISMS

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THE ROLE OF SALIVARY AGGLUTININ AND STATHERIN IN THE DEFENCE AGAINST MICRO-ORGANISMS

ACADEMISCH PROEFSCHRIFT

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Voor mijn vrouw Sandra, mijn kinderen Julia, Levi, Romy, mijn moeder Helene en mijn vader Sigfried.

Contents

Chapter 1

General Introduction

Mucosal surfaces

Mucosal surfaces are the largest and most important interface between the human body and its environment and they comprise a total area of approximately 300 m^2 of intimate contacts between the organism and the environment. Interaction with the environment is of vital importance for the uptake of nutrients and oxygen and excretion of waste products, but also makes the body vulnerable for infection and damage (Tlaskalova-Hogenova *et al.*, 2005). The external environment may have large quantities of antigenic, mitogenic and toxic stimuli in food and in the air together with pathogenic bacteria and viruses as well. To overcome these continuing challenges the immune system has developed extensive innate and adaptive responses.

The oral cavity is a part of the mucosal immune system. It is covered by saliva, which harbours a similar set of protective proteins as other mucosal fluids (Schenkels *et al.*, 1995). A major role of saliva is to control the oral microbial ecosystem. This becomes manifest when the secretion of saliva is disturbed, e.g. after radiation therapy for treatment of head and neck cancer, by auto-immune diseases affecting glandular tissues, such as Sjögren's syndrome, or as a side effect of numerous drugs. Under these conditions both qualitative and quantitative changes in the oral microflora occur. Altogether, saliva is important for maintenance of the oral health. In the absence of saliva it becomes extremely difficult to maintain the oral tissues healthy, both the mucosa and the dental enamel (Nieuw Amerongen and Veerman, 2002).

Saliva secretion

Whole saliva is for the greater part composed of secretions from three pairs of major salivary glands (parotid, submandibular, and sublingual) and from numerous minor ones (labial, buccal, lingual, palatal) (Figure 1). Each type of salivary gland secretes a fluid with a characteristic protein composition, designated glandular saliva (Veerman *et al.*, 1996). At night and in rest, mainly the (sero)mucous submandibular and sublingual glands are active. Together with the numerous minor salivary glands, they are the main contributors of the salivary mucins, mucous glycoproteins, consisting of two types: the large MUC5B, and the smaller MUC7 (Nieuw Amerongen *et al.*, 1995). The large salivary mucins (MUC5B) are responsible for the characteristic visco-elastic and slimy properties of mucous saliva (Veerman *et al.*, 1989; Van der Reijden *et al.*, 1993). The smaller MUC7 is particularly involved in the interaction with micro-organisms. During daytime, also the parotid glands contribute to whole saliva, varying from 25% in resting whole saliva up to 60% in whole saliva

stimulated by mastication. In contrast to secretions from the other major salivary glands, parotid saliva, lacking mucins, is a thin water-like (serous) saliva.

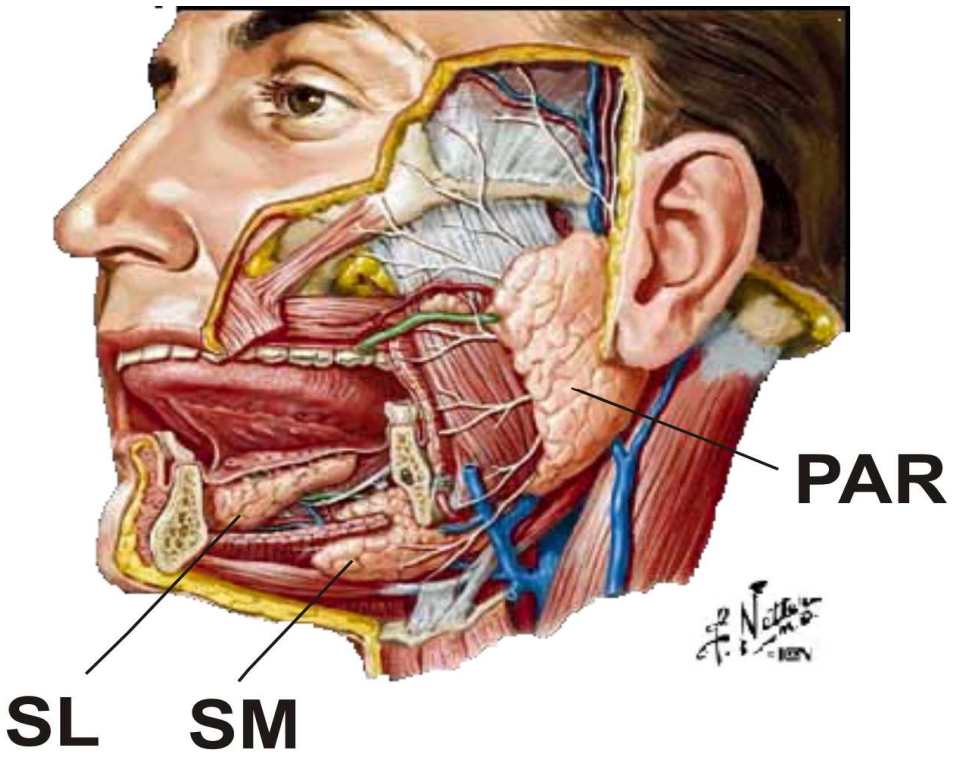

Figure 1. Location of the three major salivary glands. PAR, parotid gland; SL, sublingual gland; SM, submandibular gland. Besides these major glands there are numerous minor glands spread throughout the oral cavity, such as the palatum, cheek, lower lip. (Adapted from Netter F. H. 1959. The ciba collection of medical illustrations Vol. 3, pt 1)

Protective role of saliva

Saliva is essential for a lifelong conservation of the dentition. Without saliva the oral mucosa becomes highly vulnerable to infection and inflammation and the teeth undergo easily mechanical wear (Nieuw Amerongen *et al.*, 1987). Various functions of saliva are implicated in the maintenance of oral health and the protection of our dentition (Figure 2).

All oral surfaces are covered by a slimy layer consisting mainly of mucins. These large glycoproteins act as a diffusion barrier impeding the entry of noxious agents, including protons, micro-organisms and viruses. The mucous layer helps in reducing the friction between antagonistic tooth surfaces, and in this way

diminishes dental wear. A film of salivary proteins, called the acquired enamel pellicle, protects the tooth surface against wear. The most important pellicle proteins are, in addition to mucins, the proline-rich proteins and statherin. Proline-rich proteins (PRP) and statherin promote remineralization of calcium hydroxyapatite, the mineral phase of dental enamel, by binding calcium ions (Schlesinger *et al.*, 1987; Schlesinger *et al.*, 1989; Lamkin *et al.*, 1996), both on the dental surface and in solution.

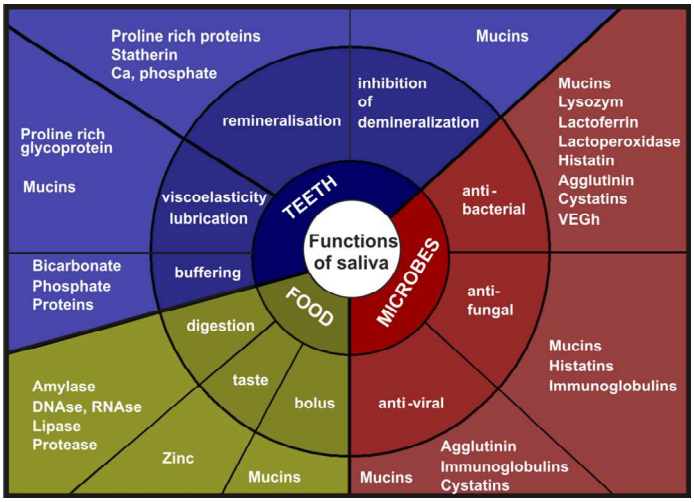

Figure 2. The various functions of saliva. (Adapted from Levine, 1993)

The role of saliva in the context of oral colonization of micro-organisms is ambiguous, since it both inhibits and supports specifically microbial colonization. Some salivary proteins directly kill micro-organisms, for instance lysozyme, histatins, defensins and the cathelicidin LL-37, or inhibit their growth, like cystatins and lactoferrin (Helmerhorst *et al.*, 1997; Edgerton *et al.*, 2000; Abiko *et al.*, 2002; Murakami *et al.*, 2002; Woo *et al.*, 2003). On the other hand, salivary proteins can serve as a nutrient source for micro-organisms. Especially the carbohydrate side chains of MUC5B, comprising up to 90 % of its molecular mass, are used as a nutrient source. Due to the heterogeneity of these carbohydrate side chains a large set of enzymes is required for complete degradation. Since each micro-organism only produces a limited set of enzymes a consortium of micro-organisms is necessary to use the full potential of mucins as a nutrient. In this way, the heterogeneity of the oral microflora may be modulated by saliva (van der Hoeven *et al.*, 1990; van der Hoeven and Camp, 1991).

Salivary components also enhance colonization of micro-organisms by forming receptors for bacterial adhesion on oral surfaces. In solution the very same salivary components may bind to micro-organisms thereby competitively inhibiting colonization and invasion into oral tissues. Salivary components that bind to micro-organisms, thereby agglutinating them, are MUC7, salivary agglutinin (SAG), s-IgA and proline-rich glycoprotein (Nieuw Amerongen and Veerman, 2002; Nieuw Amerongen *et al.*, 2004).

In this thesis special attention is given to salivary interaction with the opportunistic fungus *Candida albicans.*

Candida albicans

Besides bacteria, yeasts commonly inhabit the oral cavity, in particular *C. albicans*. The presence of *C. albicans* in the oral cavity of healthy individuals varies from 35 to 60% of the population, yet candidiasis occurs much less frequently and is related to several, often host-related, factors. For instance, individuals with a compromised immune system, e.g. HIV patients or patients under immunosuppressive medication, frequently suffer from *Candida* infections in various parts of the body, including the skin, oral cavity and esophagus, gastrointestinal tract and vagina. In the oral cavity *Candida* infection is sometimes visible as thrush, white/yellowish creamlike patches on the oral mucosa and the tongue.

An important property that contributes to the virulence of *C. albicans* is its ability to switch from the planktonic (yeast) to the filamentous (hyphal) growth form (Sanchez-Martinez and Perez-Martin, 2001; Whiteway and Bachewich, 2007; Cottier and Muhlschlegel, 2009; Soll, 2009). Each growth form has different properties which makes it possible for *C. albicans* to adjust to a variety of environmental conditions (Whiteway and Bachewich, 2007; Cottier and Muhlschlegel, 2009). The hyphal form is adhesive, can penetrate human mucosal and epithelial tissues and is therefore considered to be the most invasive form. The yeast form is much less adhesive and incapable of protruding through host tissue. On the other hand, it can easily be transported via the blood circulatory system promoting dissemination of the infection through the whole body (Grubb *et al.*, 2009).

Many authors make a difference between hyphae and pseudo-hyphae. Just like hyphae, pseudo-hyphae are elongated, but in pseudo-hyphae each cell-cell junction is constricted and the diameter of the cells is in the middle wider than at the ends. In contrast, hyphae cells are parallel without constrictions at cell junctions. There has been discussion about whether pseudo-hyphae are an

intermediate form between hyphae and yeasts or a physiologically distinct form. Carlisle *et al.* (Carlisle *et al.*, 2009) showed that formation of both hyphae and pseudo-hyphae is triggered by activation of the UME6 gene, a zinc finger transcription factor that regulates hyphae formation.

Several salivary proteins and peptides have been reported to interact with *C. albicans,* among which LL-37, histatins, defensins, IgA, lactoferrin, PRPs and MUC7 (Sigurdardottir *et al.*, 2006). In this thesis the mechanistic and functional aspects of the interaction between *C. albicans* and various salivary proteins, particularly salivary agglutinin and statherin, has been studied.

Salivary agglutinin

Salivary agglutinin (SAG), a high-molecular-weight glycoprotein encoded by the *DMBT-1* gene on chromosome 10q25.3-q.26.1, is the salivary counterpart of gp340, which is found in lung secretions. SAG (gp340) belongs to the scavenger receptor cysteine-rich (SRCR) superfamily of proteins, a group of cell-surface and/or secreted proteins, possessing SRCR domains. SRCR proteins have been implicated in developmental processes or in innate immune responses. In addition to SRCR domains, SAG contains two CUB domains and a Zona Pellucida (ZP) domain (Figure 3). ZP domains are generally involved in protein polymerization (Jovine *et al.*, 2005). This may also be the case for SAG which in saliva is present in a multimeric form (Oho *et al.*, 1998). The SRCR domains are separated by SRCR interspersed domains (SIDs), which are potential O-glycosylation sites due to their high serine/threonine content. The presence of numerous glycosidic side-chains on SIDS will probably force the SIDs into an extended stretched conformation. The polypeptide chain of the longest known variant of DMBT1^{SAG} is 2313 amino acids long with a mass of 260 kDa, but on SDS-PAGE the molecular mass is estimated at 340 kDa, due to glycosylation (Issa *et al.*, 2010; Ericson and Rundegren, 1983; Oho *et al.*, 1998).

SAG interacts with a broad range of micro-organisms, including streptococci, *Helicobacter pylori* (Prakobphol *et al.*, 2000), influenza viruses (Hartshorn *et al.*, 2003) and HIV (Wu *et al.*, 2004), but also with mucosal defence proteins, such as IgA (Ligtenberg *et al.*, 2004), surfactant proteins (Holmskov, 1999) and MUC5B (Thornton *et al.*, 2001). Stimulation of alveolar macrophage migration (Tino and Wright, 1999), suppression of neutrophil oxidative burst and activation of the complement system (Boackle *et al.*, 1993) further point to an important role in the regulation of inflammatory responses. The role of SAG in the innate immune defence has been explored by

investigating the SAG mediated activation of the complement system (Chapter 6).

SAG is one of the major bacteria-aggregating components in saliva and can be demonstrated on oral bacteria *in situ* (Issa *et al.,* 2010). Bikker *et al.* (Bikker *et al.*, 2002) discovered that the part of SAG responsible for the interaction with *S. mutans* was located in the SRCR domains of the protein (Bikker *et al.*, 2004). A 16-mer peptide fragment of the SRCR domains (SRCRP2) could both adhere and aggregate *S. mutans*. The question was addressed whether this domain is responsible for the broad-spectrum bacteria-binding properties of SAG, by examination of its interaction with a wide variety of oral and non-oral bacteria (Chapter 2).

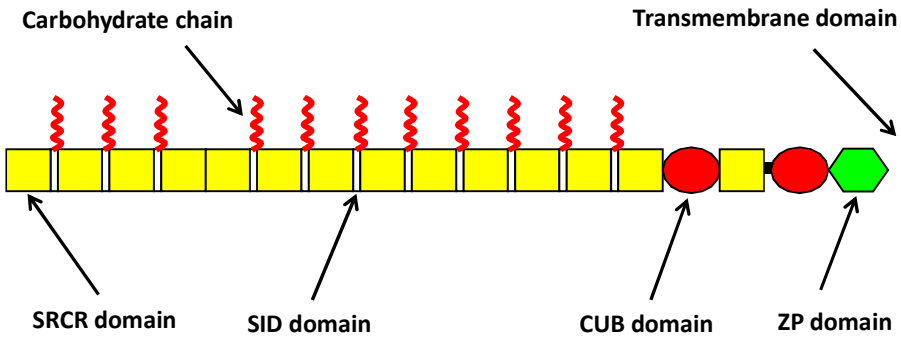

Figure 3. Structure of SAG. SAG consists of 13 SRCR domains (yellow blocks) followed by two CUB domains that are separated by another SRCR domain and then a Zona Pellucida domain. Except between the SRCR domains 4 and 5 are the first 13 SRCR domains separated by SRCR interspersed domains (SIDs) which offer many potential glycosylation sites (Holmskov *et al.*, 1999).

Statherin

Statherin is a salivary phosphopeptide of 43 amino acid residues encoded by the STATH gene on chromosome 4 (Sabatini *et al.*, 1987). Statherin is structurally disordered in aqueous solution, but NMR assignments and structural analysis in 50% trifluoroethanol water mixtures revealed three distinct structural motifs in the molecule: (1) an α-helical structure at the N-terminal domain comprising Asp1-Tyr16, (2) a polyproline type II (PPII) conformation predominantly occurring at the middle proline-rich domain spanning Gly19-Gln35, and (3) a 3^{10}-helical structure at the C-terminal Pro36-Phe43 sequence (Naganagowda *et al.*, 1998).

Statherin is implicated in the maintenance of tooth mineral. By binding calcium ions it inhibits precipitation of calcium phosphate salts and it forms a calcium buffer for remineralization. The negatively charged stretch encompassing the first five N-terminal amino acid residues (DpSpSEE) mediates binding of statherin to hydroxyapatite and inhibits crystal growth (Figure 4). The proline-rich region at the C-terminal side of the peptide has been implicated in binding to micro-organisms (Niemi and Johansson, 2004). The exact minimal binding motif, however, has yet to be identified.

Statherin coated onto hydroxyapatite surfaces has been shown to bind to a number of bacteria and fungi, including *Actinomyces spp. Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Candida albicans* (Gibbons and Hay, 1988; Gibbons *et al.*, 1988; Johansson *et al.*, 2000; Niemi and Johansson, 2004). In chapter 4 the effect of statherin on hyphae formation of *C. albicans* has been investigated.

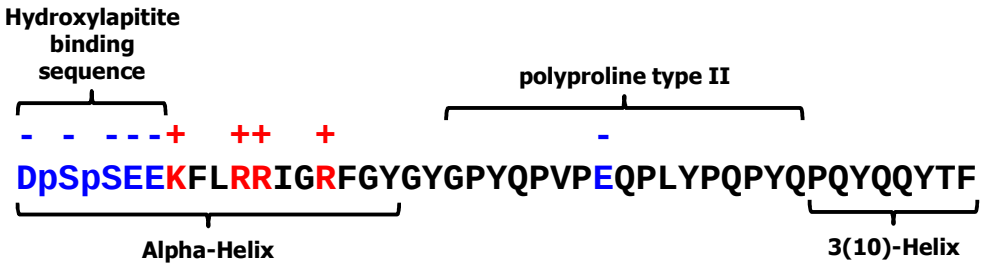

Figure 4. Amino acid sequence of statherin. Statherin consists of an α-helical structure at the N-terminal domain (Asp1 –Gly15), a middle poly-L-proline II(Gly15 – Leu29), and a β-turn at the C-terminal end (Leu29-Phe43). It contains two phosphoserines at position 2 and 3. Together with Asp1, Glu4 and Glu5 this results in a highly negatively charged N-terminus (Schlesinger and Hay, 1977).

Thesis Outline

The aim of this thesis was to study the role of saliva on the microbial colonization, especially of *C. albicans*. Salivary agglutinin and statherin were identified as two salivary substances modulating the colonization of *C. albicans.*

- In the SRCR domains of SAG the bacteria-binding site has been identified as the SRCRP2 peptide. This motif shows broad spectrum bacteria binding. In **Chapter 2** was investigated whether the binding of this peptide to different micro-organisms correlated with the binding of the whole SAG molecule. By an alanine substitution scan within this peptide was investigated which amino acids were essential for binding to different bacteria. For different bacteria different binding motifs could be identified.

- The interaction of SAG with bacteria has been subject of numerous studies. Far less is known, however, about its interaction with yeasts. In **Chapter 3** the binding of salivary components to *C. albicans* was investigated. Next to SAG also statherin was shown to bind to *C. albicans*. In contrast to bacterial binding, binding of SAG to *C. albicans* was independent of Ca^{2+}ions and not affected by reduction of SAG.

- In the oral cavity *C. albicans* is primarily present in the less virulent yeast form and not in the hyphae form. In **Chapter 4** the effect of saliva on hyphae growth of *C. albicans* was investigated. Saliva induces transition of *C. albicans* hyphae to the less virulent yeast form thus demonstrating a new salivary defence mechanism. Purification and testing of synthetic peptides showed that salivary statherin is responsible for this transition.

- In **Chapter 5** we map the domain within statherin that is responsible for *C. albicans* transition from the hyphal to the yeast form.

- Although SAG in the oral cavity primarily has been described as a bacteria binding protein it also interacts with other immune proteins which suggests a role in innate immune functions. In **Chapter 6** was investigated how SAG initiates complement activation. We show that

SAG induces complement activation through the lectin pathway by binding to mannose-binding lectin.

- In **Chapter 7** an overview has been given in which the topics that have been addressed are being discussed giving a future prospective.

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CHAPTER 2

A common binding motif for various bacteria of the bacteria-binding peptide SRCRP2 of DMBT1/gp-340/salivary agglutinin

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Abstract

Salivary agglutinin (DMBT1^{SAG}) is identical to lung glycoprotein-340 and encoded by *Deleted in Malignant Brain Tumors-1*. It is a member of the scavenger receptor cysteine-rich (SRCR) superfamily, proteins that have one or more SRCR domains. Salivary agglutinin plays a role in oral innate immunity by the binding and agglutination of oral streptococci. *Streptococcus mutans* has been shown to bind to a 16-mer peptide (QGRVEVLYRGSWGTVC) located within the SRCR domains. Within this peptide, designated SRCR peptide 2, amino acid residues VEVL and W were critical for binding.

The aim of this study was to investigate binding of DMBT1^{SAG} to other bacteria. Therefore, interaction between a series of bacteria and DMBT1^{SAG}, SRCR peptide 2 and its alanine substitution variants was studied in adhesion and agglutination assays. For different bacteria there was a highly significant correlation between adhesion to DMBT1^{SAG} and adhesion to SRCR peptide 2 suggesting that SRCR peptide 2 is the major bacteria binding site. An alanine substitution scan showed that 8 amino acids were involved in binding (xRVEVLYxxSWxxxx).The binding motifs varied for different species, but the residues VxVxY and W were always present. In conclusion, a common binding motif (RVEVLYxxxSW) within the SRCR domains is responsible for the broad bacteria-binding spectrum of DMBT1^{SAG}.

Introduction

Salivary agglutinin is a 300-400 kDa glycosylated protein which is identical to lung gp-340 and DMBT1 (Deleted in Malignant Brain Tumors-1) ((Mollenhauer *et al.*, 1997; Holmskov *et al.*, 1999; Prakobphol *et al.*, 2000; Ligtenberg *et al.*, 2001). These proteins are encoded by the gene *DMBT1* on chromosome 10q25.3-q26.1. Salivary agglutinin (designated DMBT1^{SAG}) belongs to the scavenger receptor cysteine-rich (SRCR) protein superfamily.

The SRCR superfamily comprises cell-surface molecules as well as secreted proteins that are characterized by the presence of one or more SRCR domains (Resnick *et al.*, 1994; Sarrias *et al.*, 2004). The SRCR domain is a motif of about 100 amino acids with highly conserved cysteine residues. SRCR proteins are found in metazoan organisms along the entire animal kingdom ((Freeman *et al.*, 1990; Resnick *et al.*, 1994) and in protozoan parasites such as *Plasmodium* (Claudianos *et al.*, 2002). The SRCR superfamily is divided into two groups based on the number of conserved cysteine residues within the SRCR domains. Group A proteins contains SRCR domains with 6 conserved cysteine residues, group B proteins contains SRCR domains with 8 conserved cysteine residues (Resnick *et al.*, 1994). DMBT1^{SAG} belongs to the group B SRCR proteins ((Holmskov *et al.*, 1997; Mollenhauer *et al.*, 1997; Holmskov *et al.*, 1999; Prakobphol *et al.*, 2000; Ligtenberg *et al.*, 2001). DMBT1^{SAG} homologues are found in the rat (Ebnerin) (Li and Snyder, 1995), mouse (CRP-ductin) (Cheng *et al.*, 1996), rabbit (Hensin) (Takito *et al.*, 1999), and cow (bovine gallbladder mucin) (Nunes *et al.*, 1995). Molecules of this subgroup are expressed by epithelial cells in the gastrointestinal tract and the ducts of the exocrine glands and are usually secreted into mucosal fluids (Bikker *et al.*, 2002b). They are associated with host defence, *e.g.* by pathogen binding, but also have been suggested to play a role in epithelial differentiation (Mollenhauer *et al.*, 2000).

DMBT1^{SAG} has been known initially for its role in caries prevention by binding and agglutination of cariogenic bacteria in the oral cavity (Ericson and Rundegren, 1983; Carlen *et al.*, 1998). It shows calcium-dependent binding to antigen I/II polypeptides of *Streptococcus mutans* (Demuth *et al.*, 1990). In other studies DMBT1^{SAG} was shown to have a broad bacteria recognition capability (Prakobphol *et al.*, 2000). By showing that DMBT1^{SAG} is capable of inhibiting HIV-1 infectivity an even broader role in innate immunity was suggested (Wu *et al.*, 2003).

For bacterial binding several carbohydrate receptor structures on DMBT1^{SAG} have been identified (Demuth *et al.*, 1990; Ligtenberg *et al.*, 2000; Eriksson *et al.*, 2007), but also a protein sequence in the SRCR domains of DMBT1^{SAG} has been shown to bind to *S. mutans* (Bikker *et al.*, 2002a; Bikker *et al.*, 2004). The peptide derived from this protein sequence (QGRVEVLYRGSWGTVC) was designated SRCRP2. Binding studies on N- and C-terminally shifted peptides revealed that a minimal 11 amino acids sequence (GRVEVLYRGSW) within this SRCRP2 was essential for bacteria binding. An alanine-substitution-scan on this 11 amino acids sequence revealed that the amino acids GxVEVLxxxxW were important for binding to *S. mutans (Bikker et al., 2004)*. To examine whether one and the same binding motif is responsible for the broad bacteria binding spectrum of DMBT1^{SAG}, in the present study binding of DMBT1^{SAG}, SRCRP2 and its alanine-substitution-variants have been tested upon various bacterial species.
We show that even though there are some differences in binding motifs between the different bacteria there is a common set of amino acids, VEVLY and W, which are essential for binding of all bacteria tested.

Materials and methods

Solid phase peptide synthesis

Peptides were synthesized by solid phase Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry using a MilliGen 9050 peptide synthesizer (MilliGen/Biosearch, Bedford, MA), as described previously (Bikker *et al.*, 2002a).

Peptide purification—Peptides were purified by reversed-phase HPLC on a JASCO HPLC System (Tokyo, Japan). Peptides were dissolved in 0.1% trifluoroacetic acid and applied on a VYDAC C18-column (218TP, 1.0 x 25 cm, 10-µm particles, Vydac, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid. Elution was performed with a linear gradient from 20 to 45% acetonitrile containing 0.1% trifluoroacetic acid in 20 min at a flow rate of 4 ml/min. The absorbance of the column effluent was monitored at 214 nm, and peak fractions were pooled, lyophilized, and reanalyzed by reversed-phase-HPLC and by capillary electrophoresis on a Biofocus 2000 apparatus (Bio-Rad). The authenticity of the (monomeric) peptides was confirmed by MALDI-TOF-MS. For MALDI-TOF-MS, 1 volume of sample was manually mixed with 1 volume of matrix (2 mg of a-cyano-4-hydroxycinnamic acid in 1 ml 80% acetonitrile/10 mM di-ammonium citrate) in a tube from which 0.7 ml was deposited on a stainless steel MALDI plate. MALDI-TOF-MS was performed in reflectron positive mode on the 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). The acquisition laser power was set just above the threshold. Data acquisition was automated through the software, and programmed to accumulate 5000 shots per spectrum (reflectron). The instrument was externally calibrated using a calibration peptide mixture. The purity of the peptides was at least 90%.

Purification of DMBT1^{SAG}

DMBT1^{SAG} was purified as described previously (Ligtenberg *et al.*, 2000). Twenty-five ml of human parotid saliva, collected with a Lashley cup was kept on ice for 30 min resulting in the formation of a precipitate. This precipitate was collected by centrifugation at 5,000 *g* for 20 min at 4 °C, and subsequently dissolved in 1/10 volume of TBS supplemented with 10 mM EDTA. This was applied on a Sephacryl S-400 (Amersham Biosciences, Roosendaal, Netherlands) gel filtration column (15 x 490 mm) that was run in TBS. Fractions were

monitored at 280 nm. DMBT1^{SAG} migrated in the first peak fraction and purity was checked by SDS-PAGE and silver staining.

Bacteria and growth conditions

Experiments were conducted with *S. mutans* strains Ingbritt, NG8 and UA159, *Streptococcus mitis* (HG168), *Streptococcus gordonii* (HG222), *Lactobacillus acidophilus* (HG387), *Bacillus cereus* (clinical isolate), *Staphylococcus aureus* (HG386), *Salmonella typhimurium* (LT2) and *E. coli* K12. Strains were stored frozen in Protect bacterial preservers (Technical Service Consultants LTD, Bury, Lancs., U.K.) at -80 °C. For experimental purposes, bacteria were cultured on blood agar plates and grown for 48 h at 37 °C under aerobic conditions with 5% CO_2. Subsequently, a colony was picked up for inoculation of Todd Hewitt Broth (Difco Laboratories, Detroit, Michigan, USA) for the Streptococcus species and of BHI for the other species. Cultures were grown overnight at 37 °C in completely filled 100 ml flasks under aerobic conditions with 5% CO_2 without shaking. Cells were harvested by centrifugation (3,000 g for 10 min at 22 °C), washed in TBST-Ca (10mM Tris-HCl, 150mM sodium chloride, 0.1% Tween 20, pH 7.5, 1mM $CaCl_2$) and resuspended in TBST to an optical density at 700 nm (OD_{700}) of 0.9 to 1.0.

Adhesion assay (Solid phase binding assay)

Bacterial adhesion was examined using a microplate method based on the labelling of micro-organisms with the cell-permeable DNA binding probe, SYTO-13 (Invitrogen, Leiden, The Netherlands) (Bosch *et al.*, 2003). The peptides were dissolved in distilled water to a starting concentration of 200 µg/ml. Hereafter, they were 1:1 serially diluted in Fluotrac-600 microplates (Greiner, Recklinghausen, Germany). After evaporation for 16 h at 45 °C, dried plates were washed with TBST and with distilled water. Subsequently, 100 µl of a bacterial suspension (OD_{700}: 1,0) in TBST supplemented with 1mM $CaCl_2$, was added to each well and incubated for 2 h at 37 °C. Thereafter plates were washed four times with TBST using a plate washer (Mikrotek EL 403, Winooski, VT). Bound bacteria were detected using 100 µl/well of a 1 mM SYTO-13 solution in TBS. Plates were incubated in the dark at 37 °C for 30 minutes and washed three times with TBST. Fluorescence was measured in a Fluostar Galaxy microplate fluorescence reader (BMG Laboratories, Offenburg, Germany) at 488 nm excitation and 509 nm emission wavelengths. These experiments were repeated at least three times. To convert fluorescence signals

to number of bacterial cells, for each bacterial strain the number of colony forming unit was determined by culturing, and related to the fluorescence signal.

For the competitive bacteria binding assay microplates were coated with serially diluted SAG preparation. After washing, microplates were incubated with bacterial suspensions containing 200µg/ml peptide. To examine the influence of the NaCl concentration on the adhesion of bacteria to SRCRP2 adhesion assays were performed using TBST solutions with 10, 100, 150 and 300 mM NaCl. Influence of pH on the bacterial adhesion to SRCRP2 was examined using PBST, adjusted to the appropriate pH-value.

Agglutination assays

Bacterial agglutination was studied in round-bottom microplates and confirmed by microscopy. 20 µl peptide solutions were serially diluted in 96-well round-bottom microplates (Falcon, Piscataway, NJ) at final peptide concentrations of 0–100 µg/ml. Hereafter 100 µl of bacteria suspensions in TBST-Ca was added and the mixture was incubated for 2 h at 37 °C. After agglutination or sedimentation, 5 µl was taken from the bottom of the well and transferred on a microscopic slide, Gram stained and examined by light microscopy (Leica Gmbh., Wetzlar, Germany). Agglutination activity was semi-quantified by scoring the lowest concentration at which the peptide was still able to agglutinate the bacteria completely.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SRCRP2	**Q**	**G**	**R**	**V**	**E**	**V**	**L**	**Y**	**R**	**G**	**S**	**W**	**G**	**T**	**V**	**C**
SRCRP2 **G2A**	Q	**A**	R	V	E	V	L	Y	R	G	S	W	G	T	V	C
SRCRP2 **R3A**	Q	G	**A**	V	E	V	L	Y	R	G	S	W	G	T	V	C
SRCRP2 **V4A**	Q	G	R	**A**	E	V	L	Y	R	G	S	W	G	T	V	C
SRCRP2 **E5A**	Q	G	R	V	**A**	V	L	Y	R	G	S	W	G	T	V	C
SRCRP2 **V6A**	Q	G	R	V	E	**A**	L	Y	R	G	S	W	G	T	V	C
SRCRP2 **L7A**	Q	G	R	V	E	V	**A**	Y	R	G	S	W	G	T	V	C
SRCRP2 **Y8A**	Q	G	R	V	E	V	L	**A**	R	G	S	W	G	T	V	C
SRCRP2 **R9A**	Q	G	R	V	E	V	L	Y	**A**	G	S	W	G	T	V	C
SRCRP2 **G10A**	Q	G	R	V	E	V	L	Y	R	**A**	S	W	G	T	V	C
SRCRP2 **S11A**	Q	G	R	V	E	V	L	Y	R	G	**A**	W	G	T	V	C
SRCRP2 **W12A**	Q	G	R	V	E	V	L	Y	R	G	S	**A**	G	T	V	C

Table 1. SRCRP2 alanine-substitution-variants

This table shows a schematic view of the alanine in each SRCRP2-substitution-variant synthesized. The one-letter code is used for amino acid residues. The first column shows the codenames of the peptide. The second row in the table shows the base-peptide SRCRP2. The bold printed letters in the name of each substitution-variant depicts the residue substituted and its position.

Results

DMBT1^{SAG} and SRCRP2 correlate for bacteria binding

Binding of bacteria to serial dilutions of SRCRP2 was tested in the adhesion assay. Figure 1 shows adhesion of *S. mutans* UA159, *S. gordonii* HG222, *B. cereus* and *L. acidophilus* HG387 to SRCRP2 coated plates. At a peptide concentration of 100 g/ml there was a clear difference between strongly and weakly adhering bacteria.

In the adhesion assay different bacterial strains were compared for binding to DMBT1^{SAG} and the SRCRP2 peptide (Figure 2). Both DMBT1^{SAG} and SRCRP2 mediated binding of all bacteria tested. No binding occurred to the uncoated wells. There was a highly significant positive correlation between adhesion to DMBT1^{SAG} and adhesion to SRCRP2 suggesting SRCRP2 is an important binding site on DMBT1^{SAG}.

Binding profile based on adhesion test

Within SRCRP2 an 11 amino acid sequence (GRVEVLYRGSW) has been identified as the essential binding site for *S. mutans* (Bikker *et al.*, 2004). To investigate to which extent each of the eleven amino acids contributes to bacterial adhesion, an alanine substitution scan was performed in which each amino acid across the 11 amino acid sequence consecutively was substituted by an alanine (Table 1). These peptides together with the original SRCRP2 were tested in the adhesion assay upon binding of each bacterium species (Figure 3).

The binding profiles resulting from the adhesion assays show that there is a similar trend for all the bacteria. The alanine substitutions from position 3 to 8 (RVEVLY) led to a substantial decrease in the adhesion of most bacteria. Also alanine substitution of W12 almost completely abolished the adhesion of all bacteria species tested. Replacement of glutamic acid at position 5 by alanine strongly decreased binding of streptococci, but only had a small effect on the binding to *S. aureus*, *B. cereus* and *E. coli.* Substitution of the two glycines, at position 2 and 10 leads in most cases to an increased bacterial adhesion.

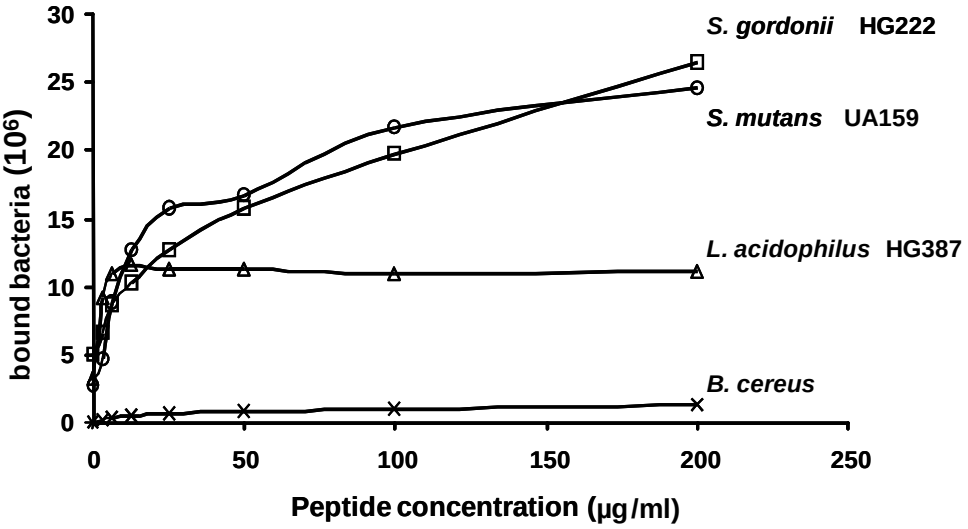

Fig. 1. Bacterial adhesion to SRCRP2
Microplates coated with SRCRP2 peptide were incubated with suspensions of bacteria. Bound bacteria were detected with a cell-permeable DNA binding fluorescent dye (syto13) and subsequently the fluorescent signal was converted in bacteria numbers. Bacteria showed large variation in adhesion. These experiments were performed with all the bacteria, at least in triplicate.

Competition assay

To examine whether SRCRP2 was able to inhibit bacteria binding to DMBT1^{SAG} a competition assay was performed with *S. mutans* and *L. acidophilus*. Microplates were coated with DMBT1^{SAG} and incubated with bacteria in the presence of SRCRP2 or DMBT1^{SAG}. Adhesion of bacteria to SAG coated plates was used as a control and set at 100%. Just like DMBT1^{SAG}, SRCRP2 inhibited the binding to DMBT1^{SAG}-coated microplates suggesting this peptide fragment is an important bacteria binding site (Figure 4).
In addition, to investigate whether for competitive inhibition the same residues were critical as for adhesion the alanine substitution variants of SRCRP2 were also tested for inhibition. The profiles obtained (Figure 4) revealed the same trend as was found in the direct binding assays, i.e. substitution of residues 2 and 10 had little if any effect, while substitution of 5,6,7 and 8 abolished inhibition almost completely.

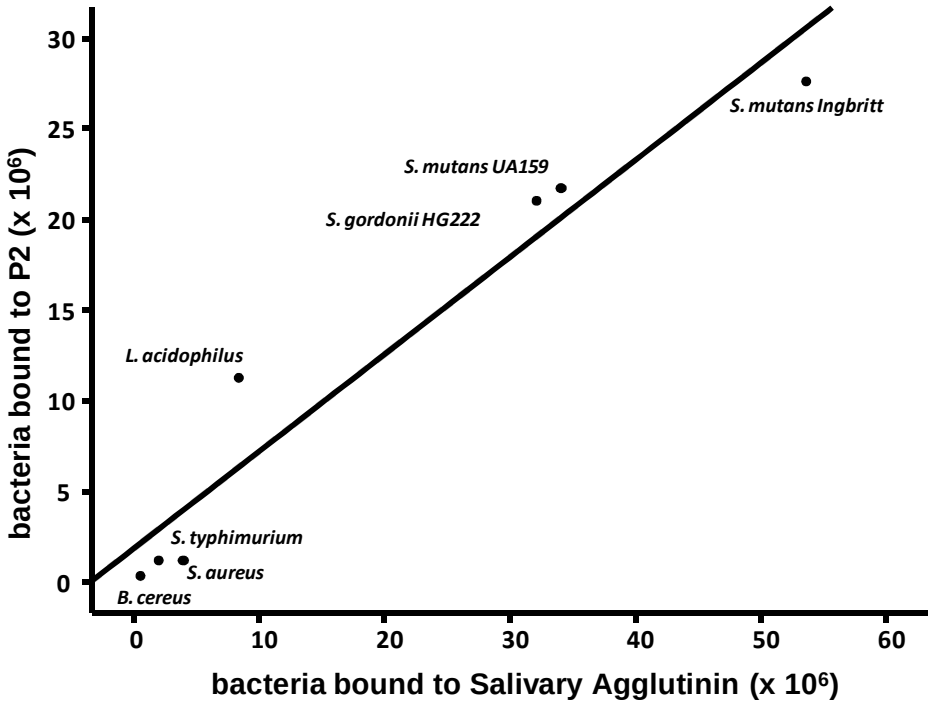

Fig. 2. Correlation between DMBT1^{SAG} and SRCRP2 for adhesion of several bacteria. SRCRP2 or purified DMBT1^{SAG} were immobilized onto microplate wells and tested for bacterial binding in the adhesion-assay. The X-axis shows the number of bacteria bound to the DMBT1^{SAG} coated wells and the Y-axis shows number of bacteria bound to the SRCRP2 coated wells. Statistical analysis was done with SPSS, using a 2-tailed Spearman rank test to calculate the correlation coefficient. The correlation coefficient was 0,964 with a significance level of 0,0005 and a linear r^2 of 0,932.

Bacteria agglutinating properties

In addition, the bacteria-binding properties of SRCRP2 and its alanine substitution variants were also tested in the soluble phase, by monitoring bacteria agglutination properties (Figure 5). Agglutination was analyzed visually and microscopically (Figure 5). When no agglutination occurred bacteria formed a white pellet in the centre of the round-bottom well (Figure 5-A2) and microscopically bacteria were homogeneously dispersed (Figure 5-A1). When complete agglutination occurred a film of bacteria was formed covering the whole bottom of the well (Figure 5-B2) and microscopically large solid aggregates were visible (Figure 5-B1). When incomplete agglutination occurred, a faint white pellet as well as a film could be detected in the well (Figure 5-C2) and microscopically a mixture of the aforementioned microscopic images was visible (Figure 5-C1).

B. cereus, *L. acidophilus* and *E. coli* K12 showed auto-agglutination. *S. typhimurium* and *S. aureus* showed no agglutination with SRCRP2 or DMBT1^{SAG}. For the other bacterial strains DMBT1^{SAG} and SRCRP2 clearly induced agglutination, highlighting the bacteria-peptide interaction in the soluble phase. Agglutination was quantified by scoring the lowest agglutinating peptide concentration (Table 2). This revealed that, with the exception of Arg-3, all residues that were critical for peptide mediated bacterial adhesion (Figure 2) were also involved in the peptide-mediated agglutination.

Gram	Bacterium	P2	G2A	R3A	V4A	E5A	V6A	L7A	Y8A	R9A	G10A	S11A	W12A
+	***S. mutans* UA159**	**100**	**50**	**100**	0	0	0	0	0	**100**	**50**	**200**	0
+	***S. mutans* Ingbritt**	**100**	**50**	**100**	0	0	0	0	0	**100**	**25**	0	0
+	***S. mjutans* NG8**	**100**	**100**	0	0	0	0	0	0	0	**100**	0	0
+	***S. gordonii* HG222**	**100**	**100**	**200**	0	**200**	0	**200**	0	0	**100**	**200**	0
+	***S. mitis* HG168**	**100**	**100**	**200**	0	0	0	0	0	0	**100**	0	0
+	***S. aureus* HG386**	0	0	0	0	0	0	0	0	0	0	0	0
-	***S. typhimurium* LT2**	0	0	0	0	0	0	0	0	0	0	0	0
+	***B. cereus***	**Auto-agglutination**											
+	***L. acidophilus* HG387**	**Auto-agglutination**											
-	***E.coli* K12**	**Auto-agglutination**											

Table 2. Minimal agglutination titre of SRCRP2 and the SRCRP2 alanine-substitution-variants against different bacteria

Bacteria were mixed with serial dilutions of peptides in microplates (200 g/ml starting concentration). After 2 hours of incubation the agglutination was scored. The agglutination titre (in g/ml) was defined as the highest dilution at which complete agglutination was detectable visually and by microscopy. A score of "0" was given when no agglutination was detected.

Influence of the electrostatic interactions on bacterial adhesion

To explore the involvement of electrostatic interactions on the binding of bacteria to DMBT1^{SAG}, we examined the effect of ionic strength and pH on the adhesion of bacteria to DMBT1^{SAG}-coated microplates. This revealed that variation in ionic strength did not influence the adhesion of *L. acidophilus* and *E. coli* (Table 3). The adhesion of *S. mutans* showed a small increase of approximately 20%, when the NaCl concentration was lowered from 300 mM to 10 mM, suggesting that electrostatic interactions to some extent play a role in the adhesion of this species to DMBT1^{SAG}. Varying the pH between 4.0 and 9.0 did not influence the binding of *L. acidophilus* or *E. coli* (Figure 6). Between pH 6.0 and 9.0 the adhesion of *S. mutans* was neither affected. However, lowering the pH from 6.0 to 4.0 resulted in an almost two-fold higher adhesion. Taken

together these experiments suggest that electrostatic interactions play only a minor role in the adhesion to DMBT1^{SAG}.

	Number of bacteria bound (x10^6)		
[NaCl]	*S. mutans*	*L. acidophilus*	*E.coli*
10 mM	27.60 ± 0.67	12.09 ± 0.24	1.61 ± 0.28
100 mM	23.71 ± 0.47	12.15 ± 0.36	1.84 ± 0.13
150 mM	22.90 ± 0.74	11.92 ± 0.48	1.85 ± 0.13
300 mM	21.03 ± 1.00	11.31 ± 0.16	1.82 ± 0.08

Table 3 Influence of the NaCl concentration on the adhesion of three bacteria to SRCRP2 coated plates.

Bacteria resuspensded in TBST with adjusted NaCl concentration were incubated in wells coated with 100 µg/ml SRCRP2 peptide. The number of bound cells was determined as described in the adhesion assay. These experiments were performed at least in triplicate.

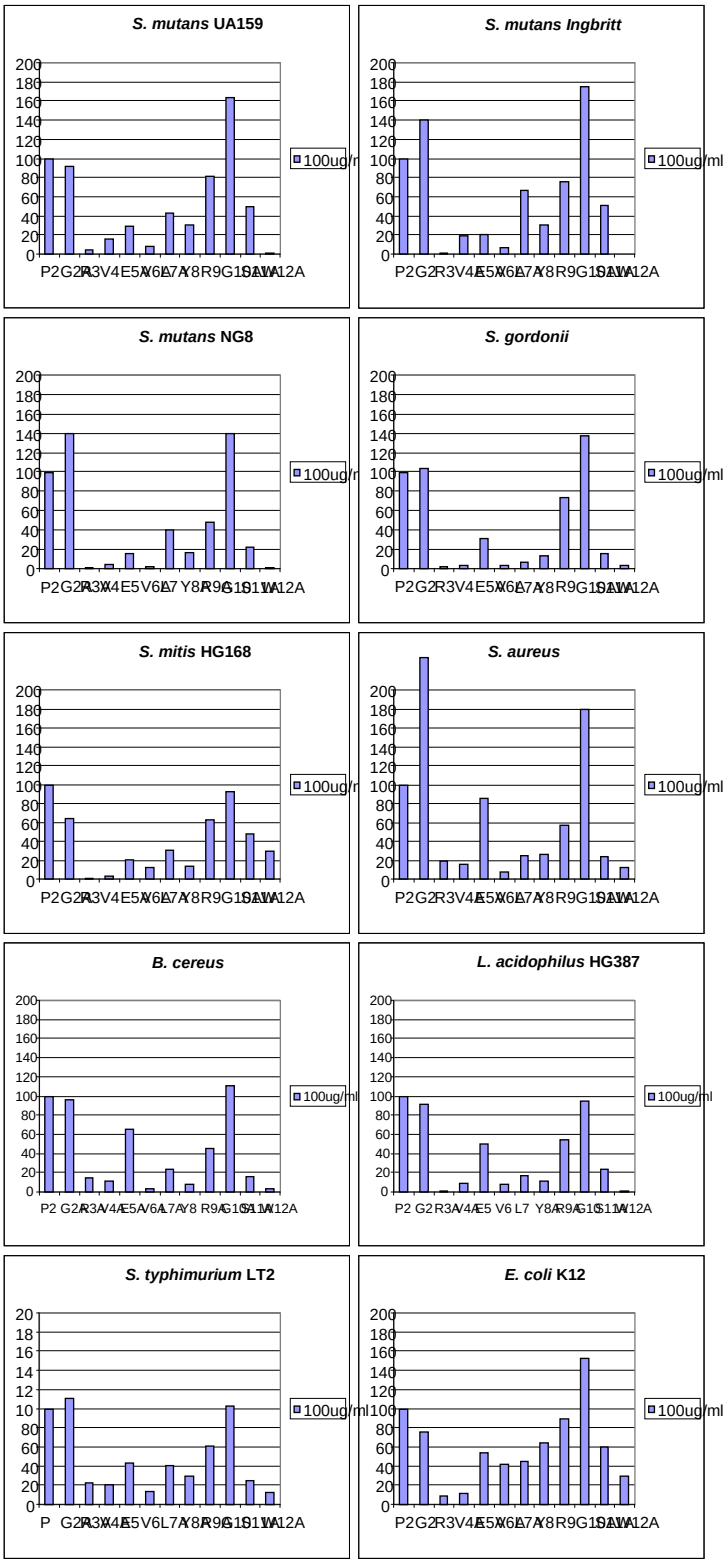
S. mutans UA159
S. mutans Ingbritt
S. mutans NG8
S. gordonii
S. mitis HG168
S. aureus
B. cereus
L. acidophilus HG387
S. typhimurium LT2
E. coli K12
100ug/ml

Figure 3. Adhesion profile of SRCRP2 peptides and alanine-substitution-variants for different bacteria

Bacteria were incubated in wells coated with 100 µg/ml of the various peptides, in the presence of the cell-permeable DNA probe SYTO-13. After washing, fluorescence was determined. For each peptide, binding is presented relative to that of the standard peptide SRCRP2 (set at 100%).

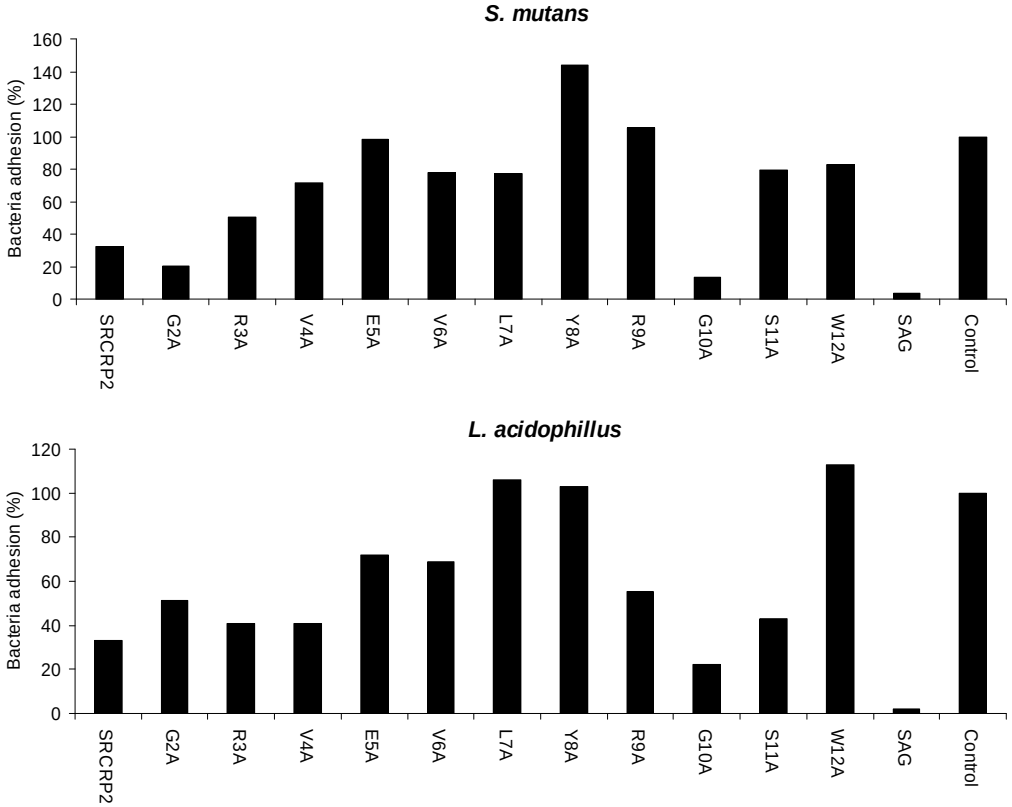

Fig. 4. Competitive bacteria binding assay with peptide to DMBT1^{SAG} coated microplates. The bacterial adhesion to SAG coated microplates was measured in the presence of peptides and compared to adhesion in buffer (100 %). As a control DMBT1^{SAG} was added to the bacteria suspension to compete with the surface coated DMBT1^{SAG}. The Y-axis shows the relative binding percentage. Peptides with high binding affinity for the bacteria were able to inhibit binding of the bacterium to the DMBT1^{SAG} coated plates resulting in a low binding percentage.

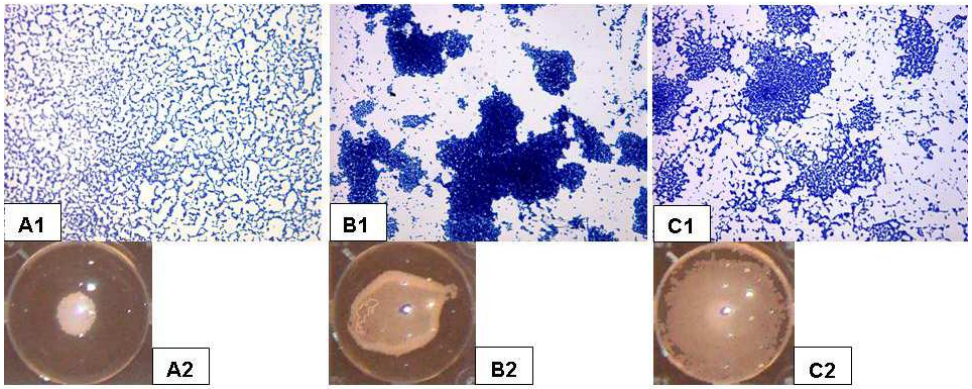

Fig. 5. Micro- and macroscopic picture of DMBT1^{SAG} induced bacterial agglutination.
DMBT1^{SAG} solution was mixed with suspension of *S. mutans* UA159 in a round-bottom micro-plate as described in Materials and Methods. After 2 hours a sample was taken and after Gram staining examined by light microscopy.
Agglutination was detected by Gram-staining (A1, B1, C1) and a visual examination (A2, B2, C2). Agglutination was visible in two forms, complete agglutination B and, incomplete agglutination C. In Figure B1 large solid aggregates are visible whereas in C1 less solid aggregates were visible. In the negative control A the bacteria were homogeneously dispersed.

Discussion

In a previous study the binding domain of DMBT1^{SAG} for *S. mutans* was identified as a 16-mer peptide within the SRCR domains (SRCRP2) (Bikker *et al.*, 2002a). In the present study a series of bacteria species was tested for binding to DMBT1^{SAG} and SRCRP2. A good correlation was found between binding to DMBT1^{SAG} and binding to SRCRP2 suggesting this peptide motif is the major binding site of DMBT1^{SAG}. Based on the adhesion assay the bacteria could be divided into two groups; bacteria with high affinity for SRCRP2 and DMBT1^{SAG} and bacteria with low affinity for SRCRP2 and DMBT1^{SAG} (Figure1). All the *S. mutans* strains, *S. gordonii* and *L. acidophilus* did bind to SRCRP2 substantially better than *S. typhimurium, S. aureus* and *B. cereus.* Both *S. mutans* and *S. gordonii* contain antigen I/II polypeptides on the cell surface which have been described for the binding of DMBT1^{SAG} (Jenkinson and Demuth, 1997). *L. acidophilus*, however, does not possess antigen I/II polypeptides suggesting another surface component on these bacteria is responsible for binding.

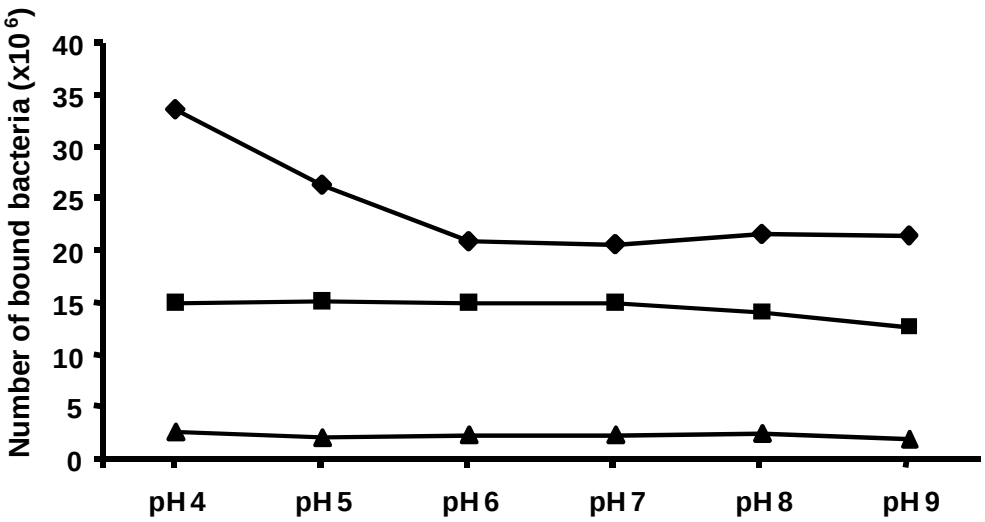

Fig. 6. Influence of the pH on the adhesion of three bacteria to SRCRP2 coated plates. Bacteria adhesion to SRCRP2 coated microplate wells was tested at different pH using pH adjusted PBST. The bacteria tested included S. *mutans* (♦) , *L.acidophilus* (■) and *E. coli* (▲). Bacteria numbers were determined as described in the adhesion assay. This experiment has been repeated three times.

Within SRCRP2 a minimal *S. mutans* binding motif of 11 amino acids has been identified in which five residues are essential (xxVEVLxxxxW) (Bikker *et al.*, 2004). In this paper the minimal binding domain for a number of other bacteria, including oral streptococci, *S. aureus*, *S. typhimurium*, *E. coli*, *B. cereus*, *L. acidophilus*, was identified by alanine substitution. Within the SRCRP2 peptide different bacteria-specific motifs were identified, but they all had 4 hydrophobic amino acids, V4, V6, Y8 and W12, in common suggesting hydrophobic interactions play a role in binding to the various bacteria species. Also the finding that adhesion of *L. acidophilus* and *E. coli* is independent of the pH and salt concentration is in line with these results. Still, substitution of these residues by alanine only slightly decreases the overall hydrophobicity, but has a profound effect on the bacteria-binding properties of the resulting variant peptide. This suggests that the interaction requires the presence of specific molecular structures, and is not merely a product of non-specific hydrophobic interactions. Depending on the bacterial species involved, additional residues are required. For instance, the interaction with all streptococci was drastically diminished when glutamic acid on position 5 was replaced by alanine. This suggests the involvement of electrostatic interactions, which is supported by the result that *S. mutans* adhesion to SRCRP2 is pH and salt dependent. This substitution had less drastic effects on the binding to *E. coli* which binds independently of the pH and ionic strength of the solution.

Surprisingly the substitution of the glycine by an alanine in the majority of cases improved the adhesion as well as the agglutination of the bacteria. In addition, substitution of an arginine at position 9 by alanine did not reduce binding whereas a substitution by glycine did (data not shown). Glycine residues generate random coil structures which are more flexible than the helix structures that are both generated by arginine and alanine. This suggests that reduction of conformational flexibility also influences the peptide bacteria interaction. According to the peptide helix prediction program AGADIR(EMBL) the G10A substitution enhanced the helix content thus possibly favouring the binding conformation of the peptide and an R9G substitution reduced the helix content resulting in reduced binding. These predictions suggest that not only the hydrophobicity but also the conformation of the peptide plays an important role in binding.

The agglutination assay with the alanine substitution variants generally confirmed the results of the adhesion assay. Only substitution of arginine at position 3, which resulted in a strong decrease in adhesion, showed much less effect on bacterial agglutination. Differences in binding of oral streptococci to

soluble phase and solid phase DMBT1^{SAG} have been described previously (Loimaranta *et al.*, 2005). This is the first study showing that SAG derived peptides show differences in interaction depending on whether they are fluid phase or surface bound.

SRCR domains have been implicated in a number of other ligand binding interactions, e.g. the binding of CD6 to activated leukocyte cell adhesion molecule (ALCAM), and the binding of MARCO and Spα to bacteria (Bowen *et al.*, 2000; Brannstrom *et al.*, 2002; Sarrias *et al.*, 2005). Interestingly, the SRCR domain of Spα contains different subdomains for LTA, and LPS, which are the ligands for Spα on Gram-positive and Gram-negative bacteria (Sarrias *et al.*, 2005).

In conclusion, the SRCR domains of DMBT1^{SAG} are important binding sites for bacteria. A core motif of four hydrophobic amino acids (V-4, V-6, Y-8 and W-12) in the SRCRP2 peptide is involved in binding to a wide series of bacteria. Other residues (R3, E-5, L-7 and S-11) may have some effect on the binding depending on the bacterial species tested.

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CHAPTER 3

Interaction between salivary agglutinin and *Candida albicans*

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Manuscript in preparation

Abstract

Salivary agglutinin (SAG), a member of the scavenger receptor cysteine-rich (SRCR) superfamily, is an antimicrobial protein found in human saliva. It plays a role in the innate immune defence of the oral mucosa by binding calcium-dependently to bacteria through its SRCR domains. *Candida albicans* is an opportunistic pathogen causing infections in immunocompromised people, xerostomia patients and denture wearers. Subject of this study is the interaction of SAG with *C. albicans*. Binding of SAG was studied by an in solution assay combined with immunoblotting, agglutination assays and adhesion assays to SAG-coated microtiterplates. In contrast to the binding of SAG to bacteria, which disappeared after chemical reduction of SAG, no effect on its binding to *C. albicans* was observed. Purified SAG agglutinated *C. albicans* in a Ca^{2+}-dependent manner. Ca^{2+}-ions could be replaced by Zn^{2+}-ions, but not by Mg^{2+}- or Ba^{2+}-ions. Unlike bacterial adhesion *C. albicans* adhesion to SAG-coated microplates was not Ca^{2+}-dependent. These results suggest that the characteristics of SAG binding to *C. albicans* are different from SAG binding to bacteria.

Introduction

Salivary agglutinin (SAG) is a 300-400 kDa glycosylated protein. Its protein sequence is identical to lung gp-340 and DMBT1 (Deleted in Malignant Brain Tumors-1) (Mollenhauer *et al.*, 1997; Holmskov *et al.*, 1999; Prakobphol *et al.*, 2000; Ligtenberg *et al.*, 2001). SAG belongs to the scavenger receptor cysteine-rich (SRCR) protein superfamily, a group of proteins containing SRCR domains which is primarily involved in host defence processes. SAG was initially found to agglutinate the cariogenic *Streptococcus mutans* upon which the bacteria are cleared from the oral cavity by swallowing. Later studies demonstrated that SAG binds to a wide variety of microbial species, including Lactobacillus *spp.,* Streptococcus *spp., Helicobacter pylori*, and viruses among which influenza viruses and HIV (Bikker *et al.*, 2002; Hartshorn *et al.*, 2003; Wu *et al.*, 2003; Leito *et al.*, 2008). SAG is believed to play an important role in the modulation of oral bacterial colonization through its broad binding spectrum and its capacity to agglutinate bacteria (Ligtenberg *et al.*, 2007).
C. albicans colonizes a variety of body locations, notably the oral cavity, genitalia and gastrointestinal tract as a benign commensal (Soll, 2002). In healthy individuals *C. albicans* does not cause infectious disease or discomfort. However, in immunocompromised individuals, individuals with impaired saliva function or denture wearers outgrowth of *C. albicans* frequently occurs.
In this study we describe a first step in characterizing the interaction between SAG and *C. albicans*.

Materials and Methods

Collection of saliva and purification of SAG

Parotid saliva from a healthy masculine donor (aged 45) was collected with a Lashley cup under stimulation by chewing on menthol chewing gum. SAG was purified as previously described (Ligtenberg *et al.*, 2000). 25 ml of human parotid saliva was kept on ice for 30 min, resulting in the formation of a precipitate. This precipitate was isolated from the saliva by centrifugation at 5,000 *g* for 20 min at 4 °C. The pellet was dissolved in 2.5 ml TBS (10 mM Tris, 140 mM NaCl, pH 7.4) supplemented with 10 mM EDTA, applied to a Sephacryl S-400 (Amersham Biosciences, Roosendaal, Netherlands) gel filtration column (1.6 x 50 cm) and eluted with TBS. Fractions were monitored at 280 nm. SAG migrated in the first peak fraction and purity was checked by SDS-PAGE using silver staining.

Chemical reduction of parotid saliva and SAG

Parotid saliva and 80 µg/ml SAG were reduced chemically by incubation with 50 mM TCEP-HCl (Tris(2-carboxyethyl) phosphine hydrochloride) (Pierce, Rockford IL, USA) for 30 min at 37 °C, according to the manufacturers protocol. Reduction was verified by ELISA and Western blotting using anti-SAG monoclonal antibody 213-06 (Antibodyshop, Gentofte, Denmark) which only recognizes unreduced SAG.

Strain and culture conditions

C. albicans 315 (ATCC 10231) was cultured aerobically at 30 °C on Sabouraud dextrose agar plates. From the agar plates a colony was picked up and inoculated in Sabouraud dextrose broth (pH 5.6) and cultured overnight at 30 °C under shaking conditions to stationary phase.

In-solution binding assay

C. albicans suspension (containing 1×10^7 cells) was centrifuged (3,000 x *g* at 22 °C for 5 min) and resuspended in 120 µl of either parotid saliva or SAG solution in saliva-buffer (2 mM potassium phosphate, 50 mM KCl, 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH 7.2). After incubation for 1 hour at 37 °C cells were harvested by centrifugation and washed twice in saliva-buffer. Next the cells were transported into a new vial and incubated in 120 µl TBS containing 2% SDS at 50 °C for 10 min for extraction yeast-bound material.

SDS-PAGE and immunoblotting

Electrophoresis was performed on NuPAGE 4-12% BIS/TRIS gels (Invitrogen, Carlsbad CA, USA). Samples were prepared by boiling for 5 min in NuPAGE LDS sample buffer (Invitrogen). After electrophoresis proteins were transferred onto nitrocellulose membranes by semi-dry blotting using an iblot gel transfer system (Invitrogen). After transfer, membranes were blocked by incubation in PBS-Tween 20 supplemented with 2 % bovine serum albumin (PBST-BSA). All subsequent incubations were performed for 1 h at room temperature in PBST-BSA. After each incubation, membranes were washed three times with PBST. Membranes were incubated with mouse monoclonal antibody 213-06 against SAG (Antibodyshop, Denmark) or biotinylated fucose-binding lectin UEA-1 (Pierce, Rockford, Il., USA). Bound 213-06 was detected with alkaline phosphatase (AP) conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark). Biotinylated UEA-1 lectin was detected with AP-conjugated streptavidin (Dako). 5-bromo-4-chloro-3-indolylphosphate (Roche Diagnostics, Mannheim, Germany) was used as a substrate.

Agglutination assays

C. albicans agglutination by SAG was studied in round-bottom microplates (Falcon, Piscataway, NJ, USA). 20 µl purified SAG (50 µg/ml) was added to 100 µl *C. albicans* suspension (approximately 1×10^7 cells/ml) in TBST (TBS + 0.1% Tween 20). As a negative control 20 µl of TBS was used instead of purified SAG. After 1 h incubation at 30 °C agglutination was determined by macroscopic and microscopic examination as described previously (Leito *et al.*, 2008).

Adhesion assay

Adhesion assays were performed as described previously (Leito *et al.*, 2008). Shortly, *C. albicans* was cultured over night in Sabouraud dextrose broth. Cells were harvested by centrifugation (3,000 x *g* at room temperature for 5 min), washed twice in TBST and suspended in TBST-Ca (TBST supplemented with 2.5 mM $CaCl_2$) to a density of 1×10^7 cells/ml. Fluotrac 600 microplates (Greiner Bio One, Netherlands) were coated by overnight incubation with 10 µg/ml SAG in 0.1 mM sodium carbonate pH 9.6 at 4 °C. After coating, plates were washed twice with TBST and once with water. Subsequently, 100 µl of *C. albicans* suspension (1×10^7 cells/ ml) in TBST-Ca was added to each well and incubated at 37 °C for 2 h. After washing 100 µl of the cell-permeant nucleic

acid stain SYTO-13 (1 mM in TBS) (Invitrogen) was added to each well. Plates were incubated in the dark at 37 ºC for 1 hour and washed three times with TBST. Fluorescence was measured in a Fluostar Galaxy microplate fluorescence reader (BMG Laboratories, Offenburg, Germany) at 488 nm excitation and 509 nm emission wavelengths.

Results

Binding of purified SAG to *C. albicans*

The binding of SAG in parotid saliva and purified SAG to *C. albicans* was tested by an in-solution binding assay. Western blot analysis revealed that SAG in parotid saliva (Fig. 1A) and purified SAG (Fig. 1B) bound to *C. albicans*. Also after reduction SAG in parotid saliva and purified SAG still bound to *C. albicans* (Fig.2).

Fig. 1. Binding of SAG to *C. albicans*. Parotid saliva (PAR) and purified SAG (SAG) were incubated with *C. albicans*. Cell-bound components were extracted with 2% SDS and analyzed by Western blot using anti-SAG mAb 213-06. (1) Starting material, (2) starting material after incubation with *C. albicans*, and (3) SDS extract of *C. albicans* after incubation were analyzed by SDS-PAGE and Western blot with anti-SAG.

Agglutination of *C. albicans* by SAG

Both the calcium dependence and the calcium specificity of SAG-*C. albicans* agglutination was tested in microplates. SAG mediated agglutination of *C. albicans* in buffer with Ca^{2+} ions but not in buffer with EGTA, a chelating agens for Ca^{2+}-ions (Fig. 3A). Ca^{2+}-ions could be replaced by Zn^{2+}-ions, but not by Ba^{2+}- or Mg^{2+}-ions. Calcium dependent agglutination of *C. albicans* by SAG was confirmed by microscopical observation. SAG clearly induced agglutination in buffer with calcium ions, but not in buffer with EDTA, a chelating agent for divalent ions (Fig. 3B).

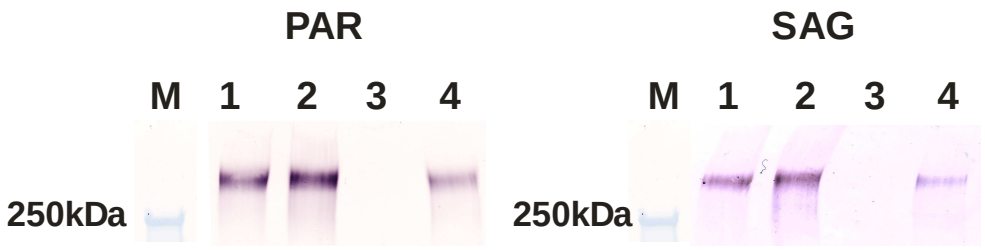

Fig. 2 Effect of chemical reduction of SAG on binding to *C. albicans*
Parotid saliva (PAR) and purified SAG (SAG) were reduced using TCEP and hereafter incubated with *C. albicans.* Cell bound SAG was extracted with 2% SDS. Samples were analyzed by Western blotting using fucose-binding lectin UEA-1. (1), starting material; (2), starting material after reduction with TCEP; (3), unbound components after incubation with *C. albicans;* (4), extract from *C. albicans* cell surface after incubation.

Adhesion of *C. albicans* to SAG-coated surfaces

C. albicans was also tested for adhesion to SAG-coated microplates (Fig. 4). Calcium dependence was tested by either adding $CaCl_2$ or EDTA to the *C. albicans* suspension. In contrast to SAG-mediated agglutination the adhesion of *C. albicans* to SAG was not significantly altered by either chelation of calcium ions by EDTA, or by the addition of extra Ca^{2+} ions (Fig. 4). To investigate the influence of pH on the interaction between *C. albicans* and SAG, adhesion was performed with *C. albicans* suspensions prepared in pH-adjusted PBS at pH 4, 5, 6, 7, 8, or 9. *C. albicans* adhesion to SAG increased with pH from pH 6 to pH 9 (Fig. 5).

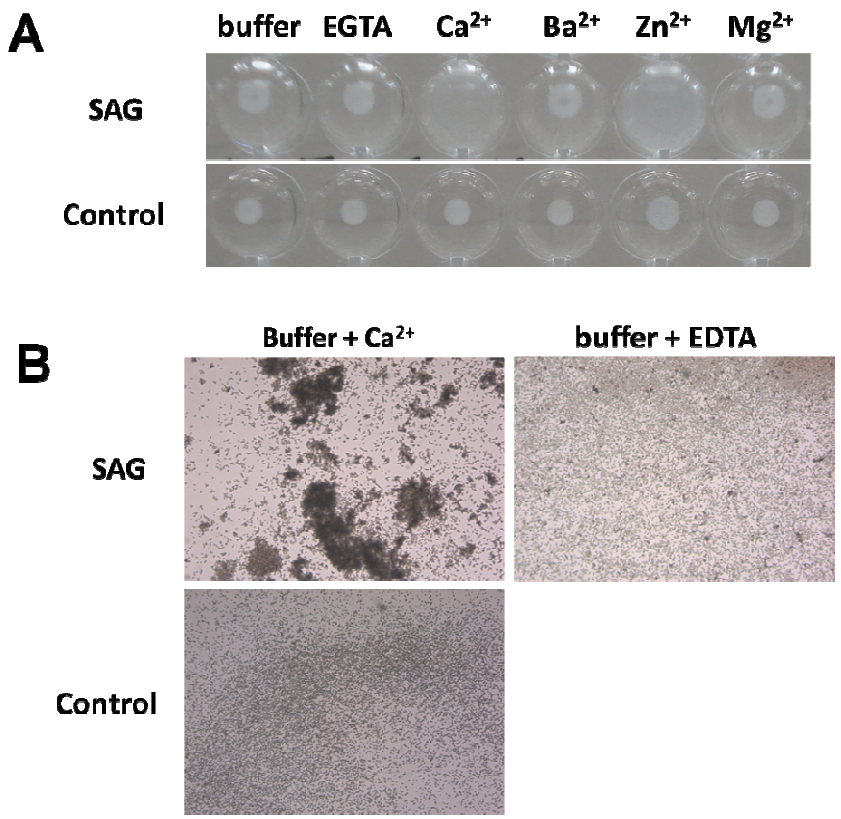

Fig. 3A. Effect of divalent cations on SAG-induced agglutination of *C. albicans*.
C. albicans was incubated with 5 µg/ml SAG in TBS in the absence or presence of 2.5mM EGTA, $CaCl_2$, $BaCl_2$, $ZnCl_2$ or $MgCl_2$. As a control *C. albicans* was mixed with the same buffers without SAG. After 1 h of incubation agglutination was visually examined. Aggregates formed were present as a thin film covering the bottom of the wells. Non-agglutinating yeast cells sedimented as a white spot in the center of the well. Ca^{2+}- and Zn^{2+}-ions enhanced SAG mediated agglutination of *C. albicans* whereas EGTA, Ba^{2+}- and Mg^{2+}-ions did not.
Fig. 3B. Microscopic view of *C. albicans* agglutination and the effect of Ca^{2+}- ions. SAG was mixed with *C. albicans* suspension in saliva buffer with $CaCl_2$ and saliva buffer with 5 mM EDTA. The control consisted of only saliva buffer. After 1 h samples were examined by phase-contrast microscopy. SAG caused the formation of large aggregates. When EDTA was added no aggregates were visible. The control showed no *C. albicans* agglutination.

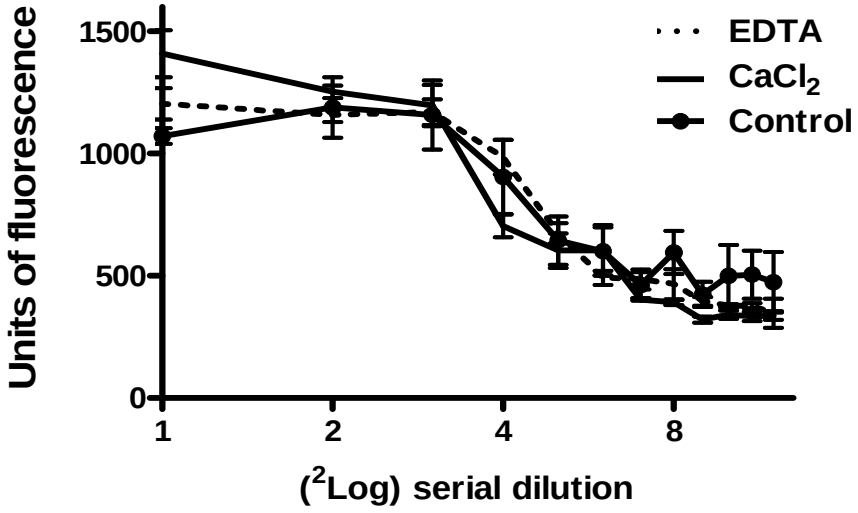

Fig. 4. Effect of calcium ions on the adhesion of *C. albicans* yeasts to SAG.
C. albicans yeast suspension in saliva buffer, saliva buffer with 2.5 mM $CaCl_2$ and saliva buffer with 2.5 mM EDTA was tested for adhesion to 3 µg/ml SAG-coated microplates. Each data point represents the mean of nine samples. Error bars represent the SEM.

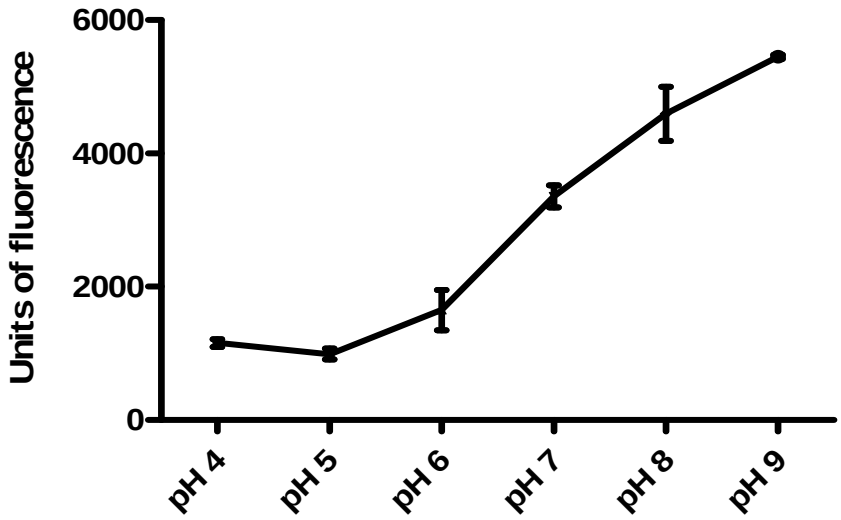

Fig.5. pH dependence of *C. albicans* adhesion to SAG.
Microplates coated with approximately 10 µg/ml SAG were incubated with *C. albicans* yeast suspension for 2 h at 37 ºC using PBS of pH 4, 5, 6, 7, 8 and 9. After washing bound *C. albicans* cells were fluorescently labeled with SYTO-13 and fluorescence was measured. Results represent mean and SD (error bars) of three experiments.

Discussion

The aim of this study was to characterize the interaction between SAG and *C. albicans*. In addition to SAG in parotid saliva also purified SAG bound to *C. albicans* (Fig. 1). It has been reported that a complex of SAG and IgA bound to *S. mutans* (Oho *et al.*, 1998). Our results show that SAG binding to *C. albicans* is not depending on interaction with other proteins such as IgA.

The reduction of SAG did not interfere with SAG binding to *C. albicans* (Fig. 2). SAG binding to *S. mutans* and other bacteria, on the other hand, is sensitive for reduction (Oho *et al.*, 1998; Ligtenberg *et al.*, 2000). Bikker *et al.*, (Bikker *et al.*, 2002) showed that a greater part of the bacteria studied bind to the SRCR domains, which contain 4 disulfide bridges. Binding is therefore sensitive to chemical reduction. *C. albicans* probably recognizes other binding sites on SAG. Other possible binding sites on SAG are the carbohydrate moieties, which have been demonstrated to bind to influenza viruses and *H. pylori* (Issa *et al.* 2010; Hartshorn *et al.*, 2003; Prakobphol *et al.*, 2005).

We found that, similar to SAG-mediated agglutination of bacteria (Ligtenberg *et al.*, 2000; Bikker *et al.*, 2004; Leito *et al.*, 2008), SAG-mediated agglutination of *C. albicans* was Ca^{2+}-dependent. Ca^{2+}-dependent binding to SAG has also been demonstrated for bacteria, IgA and surfactant protein D (Ericson and Rundegren, 1983; Holmskov, 1999; Ligtenberg *et al.*, 2000; Hartshorn *et al.*, 2006). Ca^{2+}-ions are often involved in bridging between two negatively charged surfaces. This function can be taken over by other divalent ions. Of the other ions tested, Zn^{2+}-ions could substitute for Ca^{2+}-, but Mg^{2+}- or Ba^{2+}-ions could not, suggesting that the effect involves a specific binding of Ca^{2+}- or Zn^{2+}-ions to either the protein or the yeast. In contrast, adhesion of *C. albicans* to SAG immobilized to polystyrene, occurred also in the absence of calcium in contrast to bacterial adhesion to SAG (Ligtenberg *et al.*, 2000). This also adds up to the suggestion that the *C. albicans* binding site of SAG is different from the bacteria binding site.

In conclusion, these results show that the mode of SAG binding to *C. albicans* is different from SAG binding to bacteria. Future research has to reveal the exact mechanism of SAG binding and the role in colonization of *C. albicans*.

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CHAPTER 4

Identification of salivary components that induce transition of hyphae to yeast in *Candida albicans*

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Abstract

Candida albicans, the major human fungal pathogen, undergoes a reversible morphological transition from single yeast cells to pseudohyphae and hyphae filaments. The hyphae form is considered to be the most invasive form of the fungus. The purpose of this study is to investigate the effect of whole saliva and parotid saliva on hyphae growth of *C. albicans*.

C. albicans hyphae were inoculated in RPMI medium with whole saliva, parotid saliva or buffer mimicking the saliva ion composition and cultured 18 hours at 37 °C under aerobic conditions with 5% CO_2. Whole saliva and parotid saliva induced transition to yeast growth whereas the culture with buffer remained in the hyphae form. Parotid saliva was fractionated on a reverse phase C8 column and each fraction was tested for inducing transition to yeast growth. By immunoblotting the salivary component in the active fraction was identified as statherin, a phosphoprotein of 43 amino acids that has been implicated in remineralization of the tooth surface. Synthetically made statherin induced transition of candidacidal hyphae form to the yeast form. By deletion of 5 amino acids at the negatively charged N-terminal site (DpSpSEE) the yeast form of *C. albicans* and the binding to *C. albicans* was increased. In conclusion, statherin induces transition to yeast of *C. albicans* hyphae and thus may contribute to the oral defence against candidiasis.

Introduction

The most common systemic fungal infection is candidiasis, which accounts for well over half of the invasive mycoses. A single species, *Candida albicans*, causes the majority of these infections. Its success stems in part from its capacity to live as a benign commensal in a variety of body locations, notably the oral cavity, genitalia, and gastrointestinal tract (Soll, 2002). It causes infectious disease in case the host is immunocompromised. An important feature of *C. albicans*, relevant to its pathogenesis, is its ability to switch between different morphological forms. *C. albicans* can grow in a single-celled, budding yeast form (blastospore or blastoconidia) or in a filamentous form, including both pseudohyphae and true hyphae (Berman, 2006). While yeasts are important for dissemination within the host, hyphae are essential for outgrowth and biofilm formation (Lo et al., 1997; Bastidas and Heitman, 2009). Typically *C. albicans* grows in the budding yeast form but under influence of a number of environmental signals, such as osmotic shock, alterations of temperature, pH and nutrients, it can switch to the filamentous form (Buffo et al., 1984). The hyphae form is often considered to be the essentially virulent form of the yeast (Zheng et al., 2004; Bastidas and Heitman, 2009).

Saliva contains several antifungal agents such as lactoferrin, histatin and β-defensins. Candidacidal agents like histatins have been demonstrated to kill *C. albicans* in low salt concentrations (Pollock et al., 1984; Jainkittivong et al., 1998). Histatins have also been shown to inhibit germ tube formation, but their activity was not confirmed in assays with whole saliva (Xu et al., 1991). In the present study the question is addressed if human saliva is able to induce transition to yeast growth in a hyphae growth system. We fractionated human whole saliva and parotid saliva and tested each fraction for transition to yeast growth in an RPMI based hyphae growth-system. The phosphopeptide statherin was identified as the major salivary component inducing yeast formation.

Materials and Methods

Preparation of saliva

Whole saliva of 4 healthy individuals, aged between 30 and 50 of which two women and two men, was collected without stimulation by expectoration. After collection, saliva was homogenized by shaking for 1 minute on a vortex mixer and subsequently centrifuged for 5 min at 10,000 x g to remove epithelial cells and bacteria. The supernatant was used for further studies. Parotid saliva of the same 4 individuals was collected with a Lashley cup by stimulation with menthol chewing gum. Before use, both whole and parotid saliva were filter-sterilized with a 0.45 µm pore filter (Schleicher and Schuell Biosciences, Keene, NH, USA).

Strain and culture conditions

C. albicans 315 (ATCC 10231) was cultured aerobically at 30 °C on Sabouraud dextrose agar plates. From the agar plates the yeasts cells were inoculated in Sabouraud dextrose broth pH 5.6 and cultured overnight at 30 °C under shaking conditions. To induce formation of hyphae, *C. albicans* was grown overnight in RPMI 1640 medium with 25 mM Hepes and L-glutamine (Gibco/ Invitrogen, Breda, Netherlands) 1:1 diluted in water in a shaking incubator at 37 °C under aerobic conditions with 5% CO_2.

Hyphae growth assay for *C. albicans*

C. albicans hyphae were inoculated at cell densities of 2×10^4 cfu ml^{-1} in RPMI1640 medium diluted 1:1 with either saliva or saliva-buffer which simulates the ionic composition of saliva (2 mM potassium phosphate, 50 mM KCl, 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH 7.2) and incubated for 18 h at 37 °C in 5% CO2 in 24 or 48 well micro-plates (Greiner Bio One, Frickenhausen, Germany). After incubation the cells were microscopically examined for their morphology.

Fractionation of saliva

Parotid saliva (2 ml) was fractionated by reverse phase (RP) -HPLC using a C8 column (10 x 120 mm) (Pharmacia Biotech). Elution was performed with a linear gradient of 5–45% acetonitrile containing 0.1% trifluoroacetic acid (TFA) for 72 min at a flow rate of 4 ml/min. Eluted proteins were pooled in ten fractions. The fractions were lyophilized, reconstituted in 2 ml saliva-buffer and

tested for hyphae growth inhibition. The active fraction was lyophilized, reconstituted in 2 ml HPLC-grade water, and further fractionated on a Vydac C18 column (218 TP, 10 x 250 mm, and 10 µm particles; Grace, Deerfield, IL, USA), eluted with a gradient of 10–35% acetonitrile containing 0.1% TFA in 45 min, at a flow rate of 4 ml min^{-1}. The peak fractions were again lyophilized and reconstituted in 2 ml saliva-buffer.

SDS-PAGE

Electrophoresis was performed on NuPAGE 4-12% BIS/TRIS gels (Invitrogen, Carlsbad, CA, USA). Samples were prepared by boiling for 5 min in NuPAGE LDS sample buffer (Invitrogen). After electrophoresis gels were stained with Coomassie Phastgel Blue R-350 dye (GE healthcare) and destained in 10% (v/v) acetic acid.

Immunoblotting

After electrophoresis proteins were transferred from the gel to nitrocellulose membranes by semi-dry blotting in an iBlot gel transfer system (Invitrogen). After transfer membranes were blocked by incubation in PBS-Tween 20 with 2 % bovine serum albumin. Membranes were incubated with sheep anti-statherin antibody, which was a kind gift from dr. Guy Carpenter, King's college London (Proctor et al., 2005), and hereafter with rabbit anti-sheep IgG H&L (AbD Serotec) and finally with alkaline phosphatase-conjugated anti-goat immunoglobulin (Dako). For visualization blots were incubated with 5-bromo-4-chloro-3-indolylphosphate (Roche Diagnostics, Mannheim).

Peptide synthesis

Peptides were synthesized by solid phase peptide synthesis using Fmoc chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Biosearch, Bedford, MA, USA). Purification by RP-HPLC and confirmation of authenticity by mass spectrometry were conducted as described previously (Bikker et al., 2002).

Adhesion assay

Adhesion assays were performed as described previously (Leito et al., 2008) according to the method of Bosch *et al.* (Bosch et al., 2003). After overnight culture of *C. albicans* in Sabouraud dextrose broth, yeast cells were harvested by centrifugation (3,000 x *g* at 22 °C for 5 min), washed two times in TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and suspended in TBST-

Ca (TBST with 2.5 mM $CaCl_2$) to a density of $1{\cdot}10^7$ cells/ml. The synthetic peptides were dissolved in water and coated on Fluotrac 600 microtiterplates (Greiner Bio One, Netherlands) by drying o/n at 50 °C. After coating plates were washed twice with TBST and once with water. Subsequently, 100 µl of *C. albicans* yeast cell suspension ($1{\times}10^7$ cells ml^{-1}) in TBST-Ca was added to each well and incubated at 37 °C for 2 h. After washing bound micro-organisms were detected using 100 µl per well of 1 mM SYTO-13 solution in TBS (Invitrogen, Eugene, Oregon, USA), a cell permeating and DNA-binding fluorescent probe. Plates were incubated in the dark at 37 °C for 1 hour and washed three times with the same buffer. Fluorescence was measured in a Fluostar Galaxy microtiterplate fluorescence reader (BMG Laboratories, Offenburg, Germany) at 488 nm excitation and 509 nm emission wavelengths.

In-solution binding assay

10 ml yeast cell or hyphae suspension (containing $1{\times}10^7$ cells) was centrifuged (3,000 x *g* at 22 °C for 5 min) and resuspended in 120 µl of either parotid saliva or peptide solution in saliva-buffer. After incubation at 37 °C for 1 hour cells and saliva were separated by centrifugation. The *C. albicans* pellet was washed twice in saliva-buffer. Hereafter, bound components were extracted from the *C. albicans* cells by resuspending the pellet in 120 µl TBS containing 2% SDS and incubated at 50 °C for 10 minutes. After centrifugation, the supernatants were prepared for electrophoresis.

Table 1. Amino acid sequence of statherin and statherin fragments

Ionic charge	-	-	-	-	-	+			+	+			+													-																	
statherin	D	pS	pS	E	E	K	F	L	R	R	I	G	R	F	G	Y	G	Y	G	P	Y	Q	P	V	P	E	Q	P	L	Y	P	Q	P	Y	Q	P	Q	Y	Q	Q	Y	T	F
StN6-43						K	F	L	R	R	I	G	R	F	G	Y	G	Y	G	P	Y	Q	P	V	P	E	Q	P	L	Y	P	Q	P	Y	Q	P	Q	Y	Q	Q	Y	T	F
StN20-43																				P	Y	Q	P	V	P	E	Q	P	L	Y	P	Q	P	Y	Q	P	Q	Y	Q	Q	Y	T	F
StN6-18						K	F	L	R	R	I	G	R	F	G	Y	G	Y																									

Results

Induction of yeast growth

First we determined the optimal saliva-buffer / RPMI1640 ratio to use for hyphae culture, resulting in a 1:1 dilution of saliva-buffer/RPMI. To investigate whether saliva was able to induce yeast growth an overnight culture of *C. albicans* hyphae was used to inoculate RPMI1640 medium 1:1 mixed with either saliva-buffer, parotid or whole saliva in tissue culture plates. After 17 hours of incubation in RPMI medium with whole saliva about 99% of the culture consisted of yeast cells and with parotid saliva 95% whereas in RPMI medium with saliva-buffer only hyphae were observed (Figure 1). Parotid saliva of four other subjects was tested and showed similar effects.

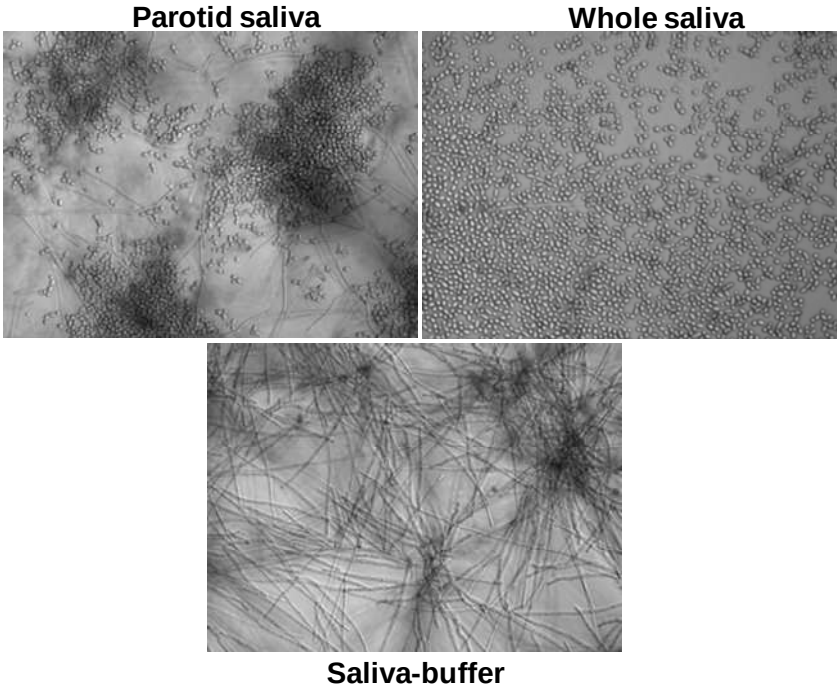

Fig. 1 Effect of different types of saliva on hyphae formation.
C. albicans hyphae were inoculated at a concentration of 1×10^4 cfu per ml in RPMI medium mixed 1:1 with whole saliva, parotid saliva or saliva-buffer. Incubation was performed for 16 hours at 37 °C and 5% CO_2 in tissue culture plates. Samples were analyzed by light microscopy at 100 x magnification. Both whole saliva and parotid saliva induce transition to yeast growth.

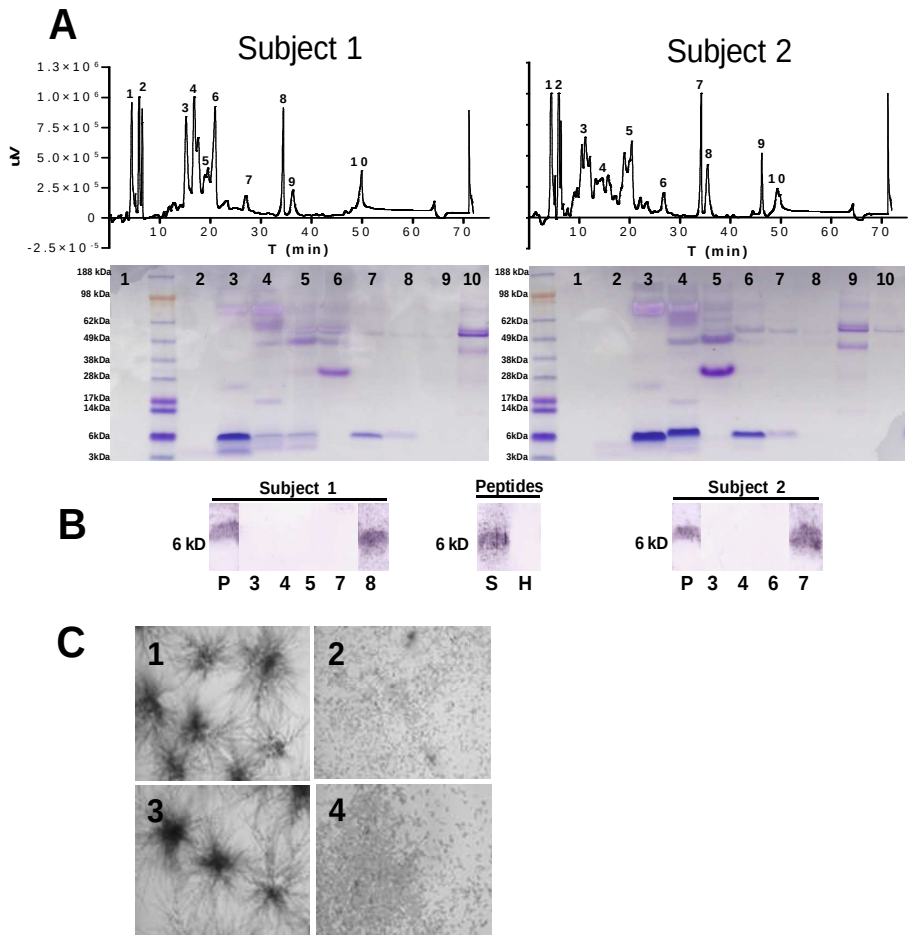

Fig. 2. Identification of statherin as the factor responsible for induction of yeast growth. (a) Two milliliters of parotid saliva was applied on a C8 RP column. Proteins were eluted as described in Materials and methods. The numbered peak fractions were collected separately, run on 4–12% Bis-Tris gels and stained with Coomassie PhastGel Blue R-350. (b) Immunoblot analysis of parotid saliva and parotid saliva fractions from subject 1 and subject 2. S, synthetic peptide statherin; H, synthetic peptide histatin 1; P, parotid saliva. Fraction 8 of subject 1 and fraction 7 of subject 2 displayed immunoreactivity with anti-statherin. Fractions 3, 4, 5 and 7 (subject 1) as well as fractions 3, 4 and 6 (subject 2) were negative. (c) HPLC fractions were lyophilized and solved in 2 mL of saliva buffer. *C. albicans* hyphae were inoculated at a concentration of 1 x 10^4 CFU/mL in RPMI medium that was mixed 1:1 with saliva buffer (1), parotid saliva (2), HPLC fraction 4 (3) and HPLC fraction 8 (4).Incubation was carried out for 16 h at 37 °C and 5% CO_2 in tissue culture plates. Samples were analyzed by light microscopy at 100 x magnification. Of the 10 fractions collected, only fraction 8 induced transition from hyphae to yeast. As an example, representative of the inactive fractions, the result obtained with fraction 4 is shown.

Purification of the hyphae inducing component

To investigate which components in saliva were responsible for the transition from hyphae to yeast growth, parotid saliva of two subjects was fractionated by HPLC on a C8 reverse phase column and peak fractions were directly analyzed by SDS-PAGE (Figure 2 A). All peak fractions were tested for induction of yeast growth. Fraction 8 of subject 1 and fraction 7 of subject 2, which migrated at the same elution time, were the only fractions that induced yeast growth (Figure 2 C4). SDS-PAGE analysis of these fractions revealed the presence of a weakly staining protein band at an apparent molecular mass of approximately 6 kDa. Considering the absorbance in the HPLC, this band showed weak staining with Coomassie Brilliant Blue. For instance fraction 7 (subject 1) which on basis of the HPLC profile contained much less protein, produced a much more intensely stained band than fraction 8 (subject 1). Based on the molecular mass of the band in fraction 8 and 7, respectively, statherin or histatin 1, which in parotid saliva are present in substantial concentrations, were predicted as possible candidates. Immunoblot analysis of these fractions with anti-statherin antibody revealed that the major band in the active fraction was statherin (Figure 2 B). Other fractions (e.g. fraction 3, 4, 5, and 7 of subject I), which migrated in SDS-PAGE at the same position, but had different retention times in HPLC chromatography, were negative (Figure 2 B). The moderate Coomassie staining of the statherin bands in fraction 7 and 8 (Figure 2) might be due to its high content of proline residues, which weakly bind to Coomassie Brilliant Blue. Spiking fraction 8 with synthetic statherin and histatin 1, respectively, confirmed that synthetic statherin co-migrated in HPLC with the major peak of the active fraction, whereas synthetic histatin 1 eluted at a different position (Figure 3 C, E). To establish further the identity of the active component, statherin and histatin 1 were synthesized and tested for their effect on hyphae growth (Figure 4). This revealed that of these peptides only statherin induced transition from hyphae to yeast, confirming again that statherin is the active component in parotid saliva.

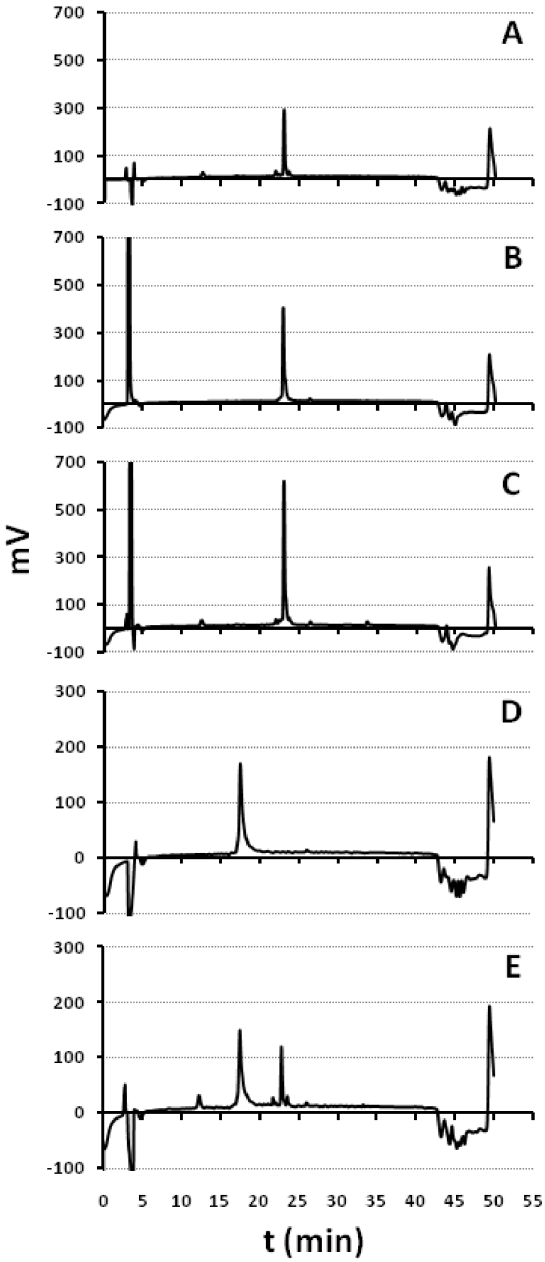

Fig. 3 Identification of the yeast-inducing factor using HPLC chromatography.
RP-HPLC chromatography on C18 of saliva fraction 8 (Figure 2) and various synthetic peptides. Fractions were dissolved in saliva buffer to a final concentration of approximately 50 μg/ml. A: fraction 8 (individual 1, see Figure 2); B: synthetic statherin; C: fraction 8 spiked with 50 μg/ml statherin; D: synthetic histatin 1; E: fraction 8 spiked with 50 μg/ml histatin 1. Statherin co-eluted within fraction 8. In contrast, histatin 1 eluted at a different position, both in saliva buffer and in fraction 8. Because a different type of chromatography column was used (C18 instead of C8), elution times differ from those in figure 2.

Mapping of the yeast growth inducing part of statherin

Statherin is a 43 residues phosphopeptide with three distinct structural motifs: an α-helical structure at the N-terminal domain (Asp_1 - Tyr_{16}), a middle proline-rich domain (Gly_{19} -Gln_{35}), and a 3_{10}-helical structure at the C-terminal Pro_{36}-Phe_{43} sequence. It contains two phospho-serines at position 2 and 3. Together with Asp_1, Glu_4 and Glu_5 this results in a highly negatively charged N-terminus which may inhibit interaction with the negatively charged *C. albicans* surface. To map the active domain a number of truncated statherin variants were synthesized (Table 1) and their effect on yeast formation tested. This revealed that the peptide StN6-43 lacking the N-terminal residues exhibited full activity (Figure 5 D).

Synthetic statherin and StN6-43 were tested for yeast transition at concentrations of 20, 10, 5, 3.5 μM. Whereas the full statherin at concentrations below 20 μM was no longer effective, StN6-43 still exhibited activity at a concentration of 3.5 μM (data not shown). The StN6-43 peptide was then further divided into two peptides, one from residue 6 to 18 (StN6-18) representing a part of the α-helical fragment and the other from residue 20 to 43 (StN20-43) representing the proline-rich part and 3_{10}-helical part of the protein. These statherin fragments were synthesized and tested for yeast growth induction (Table 1). These two peptides did not stimulate the transition to yeast growth suggesting residues in both fragments of statherin are critical for activity (Figure 5).

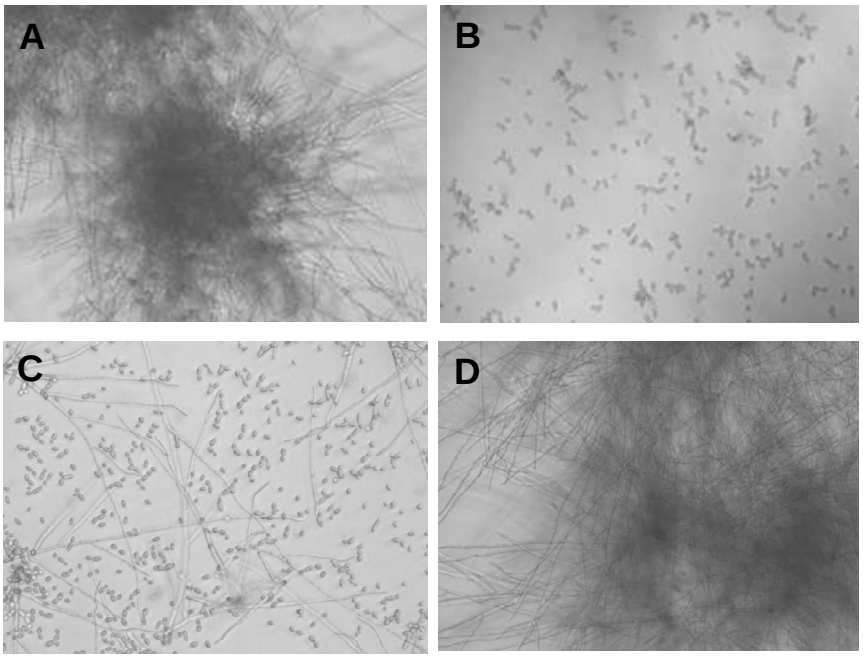

Fig. 4. Yeast growth induction by synthetic statherin and histatin 1.
C. albicans hyphae were inoculated at a concentration of 1×10^4 cfu per ml in RPMI medium mixed 1:1 with saliva-buffer (A), parotid saliva (B), 100 µM synthetic statherin (C) or 100 µM synthetic histatin 1 (D). Incubation was performed for 16 hours at 37 °C and 5% CO_2 in tissue culture plates. Samples were analyzed by light microscopy at 100 x magnification. Synthetic statherin induces transition from hyphae to yeast whereas histatin 1 did not.

C. albicans - peptide interaction

StN6-43 showed a higher yeast inducing activity than the parent statherin peptide. To test if this difference was related with the capacity of a given peptide to bind to *C. albicans* we examined their binding to *C. albicans* in an adhesion assay and an in-solution binding assay. For comparison histatin 1 was included.

For the adhesion assay microtiterplates were coated with peptides followed by incubation with a *C. albicans* suspension. Since hyphae exhibited high background adhesion to uncoated microtiterplates they could not be tested in the adhesion assay. The adhesion assay conducted with yeast revealed that StN6-43 supported adhesion of *C. albicans* better than the whole statherin molecule. Histatin 1 did not support any adhesion (Figure 6). In addition, binding of these peptides to *C. albicans* (yeast and hyphae) was tested in solution. In this assay, cells were incubated with synthetic peptides in solution.

After incubation, cells were centrifuged, washed and cell-bound components were extracted. The supernatants and the cell extracts were analyzed by SDS PAGE (Figure 7).
These experiments showed that also the in-solution-binding assay StN6-43 bound better than the whole statherin molecule, especially to the yeast form of *C. albicans* (Figure 7). SDS PAGE analysis of histatin 1 revealed a shift to a lower molecular mass position, suggesting degradation of the peptide upon incubation with the yeast form.

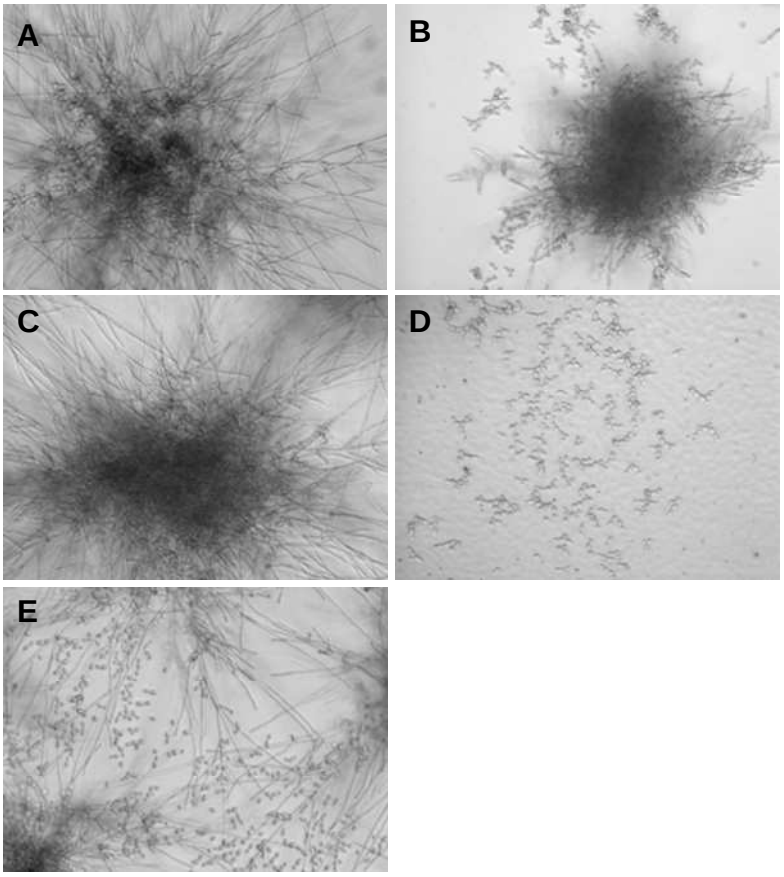

Fig. 5. Yeast growth induction by statherin and statherin fragments
C. albicans hyphae were inoculated at a concentration of 1×10^4 cfu per ml in RPMI medium mixed 1:1 with saliva-buffer as a control (A) , St6-18 (B), St20-43 (C), St6-43 (D) and full length statherin (E). All peptides where tested at a concentration of 20 µM. Incubation was done for 16 hours at 37 °C and 5% CO_2 in tissue culture plates. Samples were analyzed by light microscopy at 100 x magnification. St6-43 showed higher yeast growth inducing effect than statherin. The shorter peptides did not induce transition to yeast.

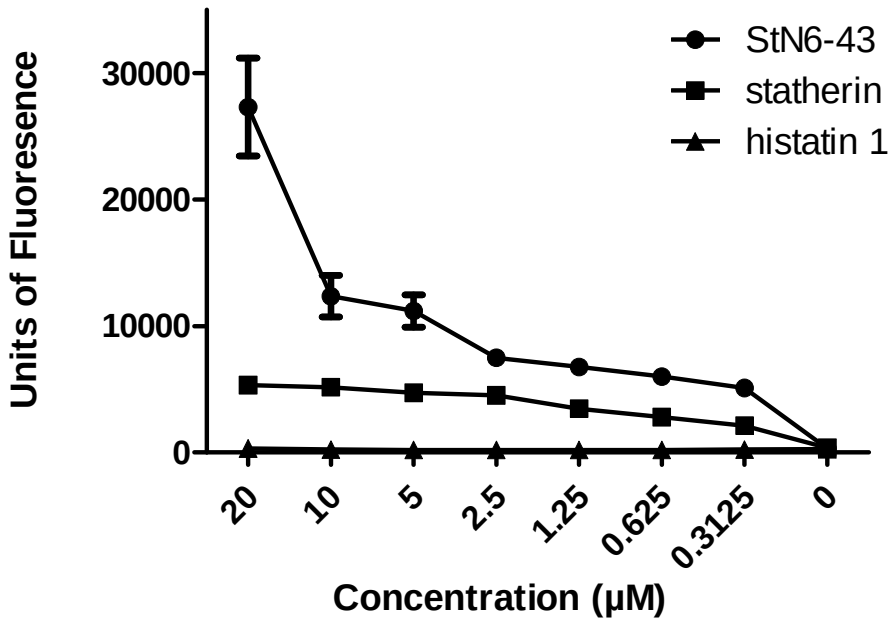

Fig. 6. Adhesion of *C. albicans* yeasts to statherin and St6-43

C. albicans yeast suspension were incubated in micro-plates coated with peptides for 2 h at 37 ºC. Unbound yeasts were washed away. Bound *C. albicans* were fluorescently labeled and the fluorescence signal was measured. *C. albicans* showed better adhesion to St6-43 than to the whole statherin. Histatin 1 did not support adhesion.

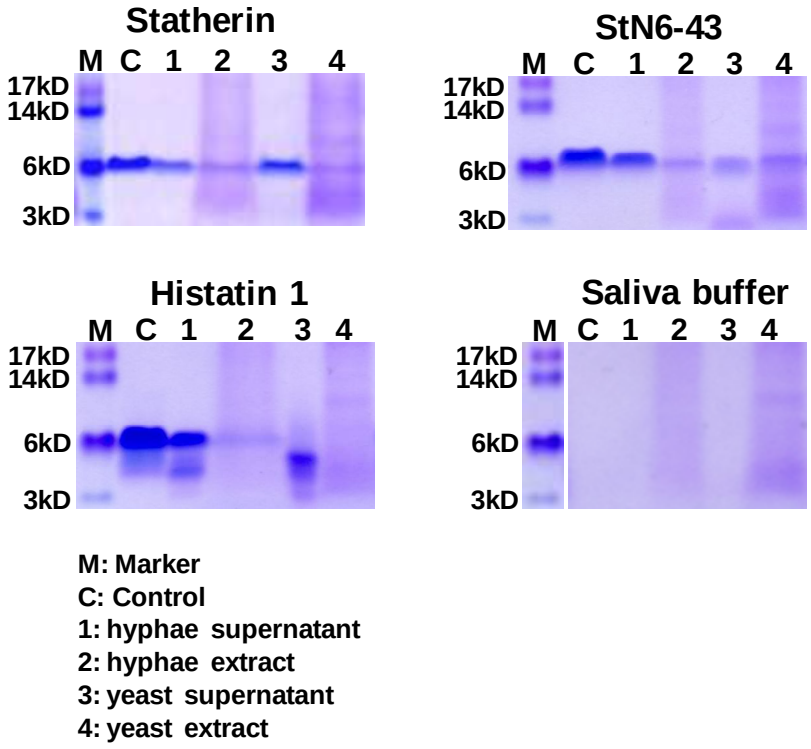

Fig. 7. In-solution-binding assay of synthetic peptides with *C. albicans*
C. albicans hyphae and yeasts were incubated in solutions of synthetic statherin, St6-43, and histatin 1 (final concentrations approximately 20 μM) for 1 h at 37 °C. After centrifugation the supernatant containing the residual unbound peptide was collected. From the pellet the cell bound components were extracted with 2% SDS. The supernatants and extracts were analyzed by SDS-PAGE. As a control for compounds originating from *C. albicans*, *C. albicans* was incubated in saliva buffer and analyzed in the same way.

Discussion

This study demonstrates that whole saliva and parotid saliva promote the transition of *C. albicans* to the yeast form in a hyphae growth system. The salivary component responsible for this transition was identified as statherin. In an earlier report synthetic histatins inhibited germ tube formation, especially histatin 3 (Xu et al., 1991). Another paper reported that human saliva inhibits germ tube formation but could not confirm the role of histatins possibly due to complexation of histatins with other salivary proteins (Xu et al., 1991; Santarpia et al., 1992). In this paper histatin 1 was also tested for inducing transition to yeast growth, but no effect was found in our system.

Statherin is a 43-residue acidic salivary phosphopeptide. Its gene is encoded on chromosome 4 and shows homology with the HIS1 gene for histatins (Sabatini et al., 1993). Statherin is secreted by the parotid and submandibular/sublingual salivary glands. The concentration of statherin in saliva is in the range of 16–160 µg/ml (Hay et al., 1984). The concentration that we used in the hyphae inhibition assay (100 µg/ml) is within this range and thus is physiologically relevant. Since the hyphal form of *C. albicans* is considered to be more infectious than the yeast form inhibition of hyphae formation may play a role *in vivo* (Lo et al., 1997; Saville et al., 2006).

Statherin is among the first salivary proteins that bind to the enamel surface and is considered to be of prime importance in protecting the teeth (Hannig et al., 2004). Coated onto the dental surface statherin binds a variety of micro-organisms, including *C. albicans* (Gibbons and Hay, 1988; Gibbons et al., 1988; Johansson et al., 2000). This was confirmed in our binding assays where we found a good adhesion of yeast cells to statherin-coated microplates. Previous research has shown aggregation of *C. albicans* by 2 mg/ml statherin in solution (Johansson et al., 2000), which is 20-fold higher than the concentrations we used (100 µg/ml) in our in solution binding assay.

Statherin shows a high electrical charge asymmetry. A significant number of negatively charged residues is localized in the N-terminal region (DpSpSEE). This is followed by a short positively charged region, whereas the C-terminal region contains mainly uncharged residues. The first five negatively charged amino acids are responsible for the coating of statherin on hydroxyapatite (Raj et al., 1992). Remarkably, when these five amino acid were absent, as in StN6-43, the yeast inducing effect was enhanced. The decreased repelling forces between the negatively charged amino acids and the negatively charged cell surface of *C. albicans* could explain this. This was confirmed by the

observation that StN6-43 compared to statherin gave a better adhesion of *C. albicans* and clearly showed binding in solution to both yeast cells and hyphae. StN6-43 on its turn was further divided into a proline-rich part StN20-43 and StN6-18 that has a strong positive charge due to one lysine and three arginine residues. Both fragments did not stimulate transition to yeast growth as effectively as the StN6-43 peptide suggesting that residues on both fragments are required for transition to yeast growth. This result is in line with the result of Niemi and Johansson who only found binding of *C. albicans* to intact statherin and not to StN1-32 or StN33-43 (Niemi and Johansson, 2004).

Since the hyphal form of *C. albicans* is considered to be more virulent than the yeast form inhibition of hyphal growth of *C. albicans* by saliva may be one of the effects by which saliva prevents the development of candidiasis in individuals carrying of *C. albicans* in the oral cavity. For people suffering from dry mouth, immuno-compromised people and people that are sensitive for Candida outgrowth in general, statherin based peptides such as StN6-43 might be an interesting additive in antifungal applications.

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CHAPTER 5

Mapping of the minimal domain on statherin that induces hyphae-to-yeast transition in *Candida albicans*

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Abstract

Candida albicans, a major fungal pathogen in humans, may undergo a reversible morphological transition from the yeast form to the more virulent hyphae form. Previously we demonstrated that statherin, a 43 residue peptide implicated in the mineral homeostasis of saliva, induces hyphae-to-yeast transition (Chapter 4). The aim of this study was to map the minimal domain of statherin that is responsible for this activity. For this purpose, truncated statherin peptides were synthesized and added to *C. albicans* hyphae cultures. Hyphae growth was scored microscopically. Cultures without statherin peptides showed only hyphae growth. Hyphae-to-yeast inducing activity enhanced by removal of the first nine N-terminal amino acids of statherin (StN10-43). By removal of another four residues (StN14-43) activity disappeared. By C-terminal truncation of StN10-43 activity decreased. StN10-29 was still active, but StN10-19 was not. Most truncated variants that induced hyphae-to-yeast transition also caused detachment of adhered hyphae from the culture plates.
These results show that fragments of statherin are able to induce hyphae-to-yeast transition in *C. albicans*.

Introduction

Candida albicans is the most widespread fungal pathogen in humans and can cause life-threatening systemic infections. *C. albicans* is a common constituent of the microflora of the gastro-intestinal tract. At these sites disturbance of the ecological balance in the mucosal flora may cause opportunistic infections with *C. albicans*, such as vaginitis or thrush. Known factors that can cause this disturbance are use of antibiotics and compromised immunity by for instance, radio-therapy, AIDS or surgery (Soll, 2002).

An important feature of *C. albicans*, relevant for its pathogenesis, is its ability to switch between different morphological forms. *C. albicans* can grow in a unicellular yeast form, known as blastospores or blastoconidia, or in filamentous structures known as pseudohyphae and true hyphae (Berman, 2006). Under influence of a number of environmental signals, such as osmotic shock, alterations of temperature, pH and nutrients, yeasts can switch to the filamentous form (Buffo *et al.*, 1984). While yeasts are important for dissemination within the host, hyphae are essential for outgrowth and biofilm formation (Lo *et al.*, 1997; Bastidas and Heitman, 2009). Compared to the yeast form hyphae show stronger binding to epithelial cells and can penetrate human tissue. Therefore, the hyphae form is considered to be the most virulent form of *C. albicans* (Zheng *et al.*, 2004; Bastidas and Heitman, 2009).

Saliva contains several antifungal agents that are able to kill *C. albicans*, such as histatins, LL-37 and β-defensins, or inhibit its growth, such as lactoferrin (Pollock *et al.*, 1984; Jainkittivong *et al.*, 1998; van der Kraan *et al.*, 2004; Matejuk *et al.*, 2010). In a previous study we revealed an alternative mechanism by which saliva controls *C. albicans* infection. In the presence of salivary statherin *C. albicans* grows in the yeast form under circumstances that normally induce hyphae growth. Statherin is a 43 residues calcium-binding phosphopeptide which, as constituent of the tooth pellicle, plays a role in the mineral homeostasis of saliva by keeping saliva supersaturated with respect to calcium phosphate.

In the present study we have mapped the minimal active domain of statherin that is responsible for the transition of hyphae-to-yeasts *of C. albicans*. For this purpose, fragments of the statherin molecule were evaluated for their ability to induce hyphae-to-yeast transition. A peptide representing residues 10-43 of statherin induced the strongest hyphae-to-yeast transition. A peptide representing the residues 6-18 was the shortest fragment that still induced hyphae-to-yeast transition.

Materials and Methods

Peptide synthesis

Peptides were synthesized by solid phase peptide synthesis using Fmoc chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Biosearch, Bedford, MA, USA). Purification by reversed phase-HPLC and confirmation of authenticity by mass spectrometry were conducted as described previously (Bikker *et al.*, 2002). Truncated statherin peptides were denoted StN with the corresponding residues in the whole protein, for example StN10-43 consists of the residues 10 to 43 of the full length statherin.

Strain and yeast cell culture conditions

C. albicans 315 (ATCC 10231) was cultured aerobically at 30 °C on Sabouraud dextrose agar plates (Oxoid, Hants, UK). From the agar plates the yeasts cells were inoculated in Sabouraud dextrose broth pH 5.6 and cultured aerobically overnight at 30 °C under shaking conditions. This culture was diluted 1:50 in fresh medium and grown for 6 h until early stationary phase.

Hyphae-to-yeast induction

C. albicans yeast cells were inoculated at densities of 50 cfu ml^{-1} in RPMI 1640 medium with 25 mM Hepes and L-glutamine (Gibco/Invitrogen, Breda, Netherlands) diluted 1:1 with H_2O. Cultures were incubated for 18 h at 37 °C under aerobic conditions with 5% CO_2 in 48 wells micro-plates (Greiner Bio-One, Frickenhausen, Germany). This condition induced the transition of yeasts to the hyphal morphology. After culturing *C. albicans* was present as clusters of hyphae attached to the bottom of the wells of the culture plate. Then medium was removed and 500 µl of the same medium containing 20 µM of peptide was added. The plates were incubated for 18 h at 37 °C and 5% CO_2. After incubation the medium was mixed with a pipet and transferred to a Neubauer counting chamber to determine the number of yeast cells that had been generated upon incubation with peptide. The yeast cells within 16 squares, corresponding to a volume of 100 nl, were counted. To determine the detaching effect of the peptides the fluid was removed and the residual number of hyphae clusters attached to the bottom of the wells was determined.

Analysis of peptide-binding to *C. albicans* .

Early stationary phase *C. albicans* culture was centrifuged and resuspended in PBS. After a second centrifugation step yeast cells were suspended in PBS to a density of 1×10^7 cells/ml. 360 µl cell suspension was supplemented with 40 µl of peptide solution in a 48 well culture plate (Greiner) to a final peptide concentration of 20 µM. Plates were incubated for 1 h at 37 °C. To control for non-specific adsorption of peptides to the microplate wells, parallel incubations were conducted with peptides alone. At the start and after 1 h a 50 µl aliquot was taken and centrifuged (10,000 x g, 5 min) for determination of the peptide concentration. The amount of peptide before and after incubation was determined by capillary zone electrophoresis (CZE) on a Bio-Focus 2000 capillary electrophoresis system (Bio-Rad, Hercules, CA) as described by Ruissen *et al.* (Ruissen *et al.*, 2002).

Binding of peptides to the hyphae form was determined essentially the same way. *C. albicans* hyphae were grown as described above for hyphae-to-yeast induction in 48 well culture plates. After 18 h medium was carefully removed by aspiration, and hyphae, attached to the bottom of the culture plate, were washed twice with PBS. Then 400 µl of a 20 µM peptide solution in PBS was added and incubation was performed for 1 h at 37 °C. At the start and after 1 hour aliquots were taken and centrifuged (10,000 x g, 5 min). Peptide concentrations in the supernatant were analyzed by CZE.

Table 1. Amino acid sequence and functional activities of the peptides used in this study

Ionic charge	− − − − − − + + + + −	[1]Yeast Growth	Hyphae detachment	[2]Yeast Binding
Statherin	D pS pS E E K F L R R I G R F G Y G Y G P Y Q P V P E Q P L Y P Q P Y Q P Q Y Q Q Y T F	++	++	+
StN6-43	K F L R R I G R F G Y G Y G P Y Q P V P E Q P L Y P Q P Y Q P Q Y Q Q Y T F	++	++	+
StN10-43	R I G R F G Y G Y G P Y Q P V P E Q P L Y P Q P Y Q P Q Y Q Q Y T F	++	++	+
StN14-43	F G Y G Y G P Y Q P V P E Q P L Y P Q P Y Q P Q Y Q Q Y T F	−	−	
StN20-43	P Y Q P V P E Q P L Y P Q P Y Q P Q Y Q Q Y T F	−	−	−
StN10-37	R I G R F G Y G Y G P Y Q P V P E Q P L Y P Q P Y Q P Q	+	++	
StN10-29	R I G R F G Y G Y G P Y Q P V P E Q P L	+	++	
StN10-19/37-43	R I G R F G Y G Y G - - - - - - - - - - - - - - - - - - Q Y Q Q Y T F	+	+	
StN10-19	R I G R F G Y G Y G	−	+	
StN6-18	K F L R R I G R F G Y G Y	+	−	+
HIS1	D S H E K R H H G Y R R K F H E K H H S H R E F P F Y G D Y G S N Y L Y D N	−	+	−
CYS1-13	S S S K E E N R J J P G G	−	−	−

[1] **++**: strong hyphae-to-yeast induction; **+**: moderate hyphae-to-yeast induction; **-**: no hyphae-to-yeast induction
[2] **++**: strong hyphae detachment; **+**: moderate hyphae detachment; **-**: no hyphae detachment

Results

Determination of the minimal active domain of statherin

We synthesized a series of statherin fragments, truncated at the N- and C-termini (Table 1). These peptides were evaluated for their potential to induce transition to yeast growth in a hyphae growth system in a concentration of 20 µM. As negative control the solvent alone was used (1:1 diluted RPMI), 20 µM histatin 1 (HIS1, 38 residues) (Leito *et al.*, 2009) and CYS1-13, a peptide representing the N-terminal 13 residues of cystatin S.

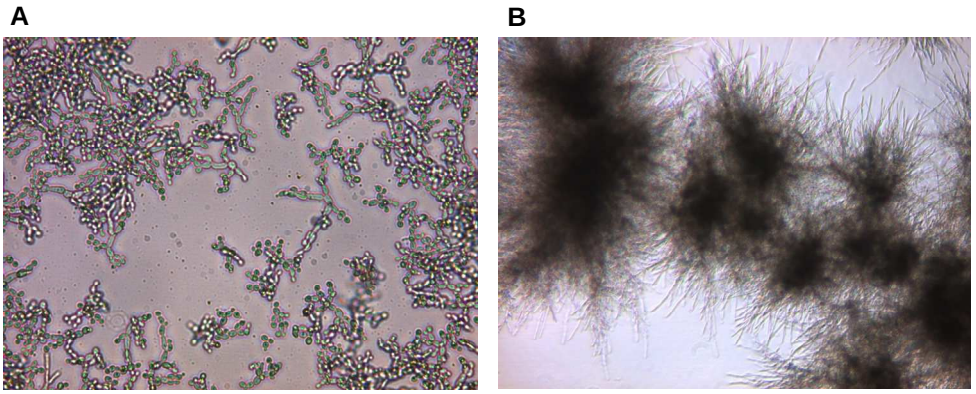

Fig. 1. Effect of statherin peptide StN6-43 on hyphae-to-yeast transition of *C. albicans*. *C. albicans* hyphae were cultured for 17 h at 37 °C with 5% CO_2 in medium with (A) or without (B) 20 µM StN6-43.

Figure 1A shows a microscopic picture obtained after incubation of hyphae with peptide StN6-43 revealing that compared to the control the number of yeast cells was increased. In the control only hyphae clusters were visible (Fig. 1B).

By deletion of the N-terminal amino acids of statherin, as in StN6-43 and StN10-43, hyphae-to-yeast inducing activity increased, with peptide StN10-43 showing the highest activity (Fig. 2). By further deletion of N-terminal amino acids, as in peptides StN14-43 and StN20-43, activity disappeared (Fig. 2). C-terminal truncation of StN10-43 resulted in a decrease of activity in StN10-37 and StN10-29. StN10-19 showed no activity, but N-terminal elongation, as in peptide StN6-18, restored its activity partially. Also elongation of this peptide with residues 37-43 (StN10-19/37-43) restored its activity.

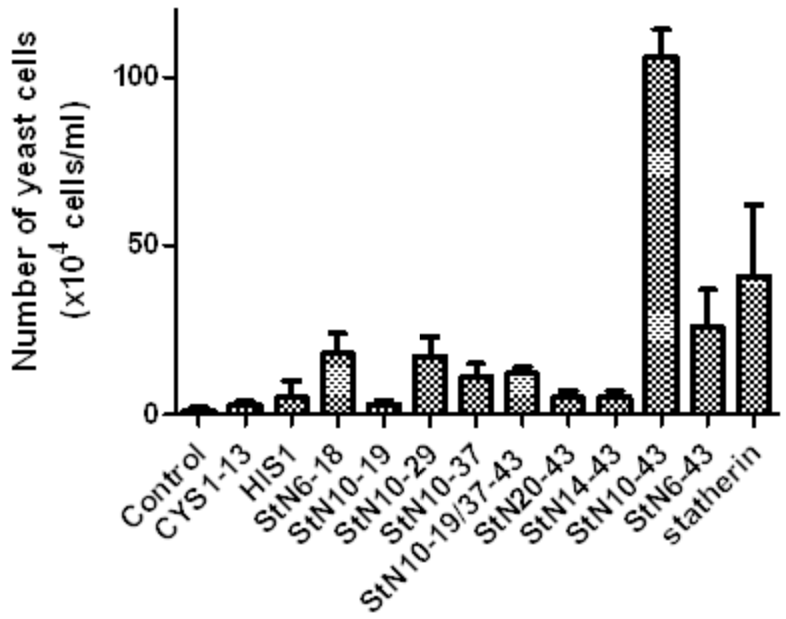

Fig. 2. Hyphae-to-yeast-inducing activities of statherin-derived peptides.
C. albicans hyphae were cultured for 20 h under hyphae-inducing conditions as described in Materials and Methods in the presence of 20 μM peptides. Yeast cells were counted using a Neubauer counting chamber. Results represent mean and SD (error bars) of three samples. Control: hyphae culture without peptides.

Detachment of hyphae clusters

We observed that peptides inducing the hyphae-to-yeast transition caused detachment of hyphae clusters from the bottom of the wells of the culture plates. Therefore, we counted the number of attached hyphae clusters before and after incubation with peptides (Fig. 3). Hyphae-to-yeast induction and hyphae detachment correlated ($p < 0.05$), but were not directly linked. E.g. StN6-18 induced hyphae-to-yeast transition, but no hyphae detachment, whereas on the other hand StN10-19 and histatin 1, while not inducing hyphae-to-yeast transition, did cause detachment of hyphae.

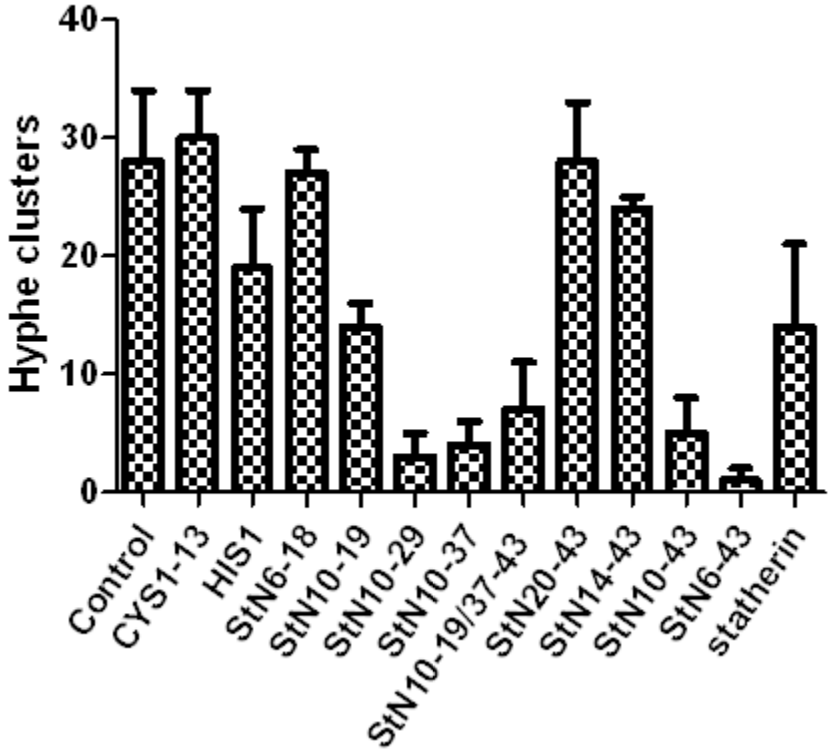

Fig. 3. Effect of statherin-derived peptides on the detachment of hyphae
C. albicans hyphae were cultured for 20 h under hyphae-inducing conditions as described in Materials and Methods in the presence of 20 μM peptide. After washing with PBS the number of hyphae clusters that remained attached to the culture plate wells was counted. The X-axis shows the tested peptides and the Y-axis shows the number of attached hyphae clusters per well. Results represent mean and SD (error bars) of three samples.

Binding of peptides to *C. albicans* hyphae and yeasts

To investigate whether there are differences in binding properties for peptides between hyphae and yeasts several peptides were evaluated for binding to both forms of *C. albicans* (Fig. 4). StN6-43 and StN10-43, which induced both yeast cell growth and hyphae detachment, bound to both yeast cells and hyphae. StN6-18, which did not induce detachment of hyphae, bound to both yeasts and hyphae. Cys1-13 did not bind to either hyphae or yeasts. These results suggest that there are no essential differences for binding of the peptides to either hyphae or yeasts.

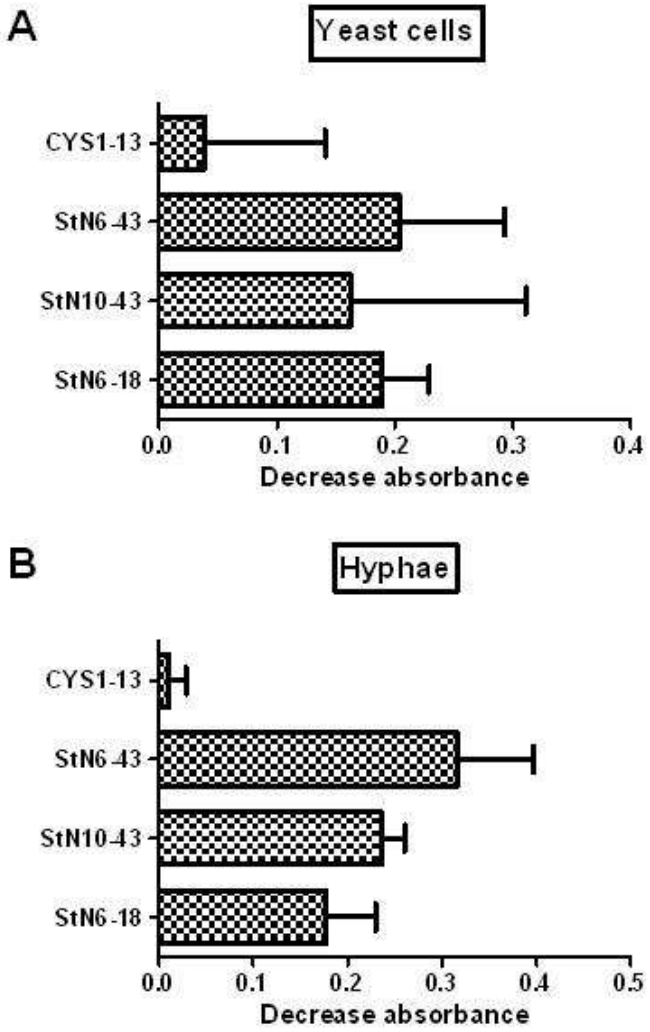

Fig. 4. Binding of peptides to *C. albicans* yeasts (A) and hyphae (B)
(A) Peptide solutions (20 µM) were incubated in 48 wells plates with (A1) or without yeast cells (A0). After 1 h samples were taken and peptide concentrations were determined by CZE with imidazole as an internal standard. The horizontal axis shows the decrease in peptide concentration compared to the control (A1 –A0).
(B) 48-wells culture plates covered with hyphae were incubated with 400 µl 20 µM peptide solution at 37 °C (B1). Peptide solutions in 48 wells plates without hyphae served as a control (B0). Samples were taken after 1 h. The peptide concentration was determined by CZE with imidazole as an internal standard. The horizontal axis shows the decrease in peptide concentration compared to the control solution (B1 – B0).

Discussion

In Chapter 4 we showed that salivary statherin is able to induce the transition of hyphae to yeast cells under hyphae growth conditions (Leito *et al.*, 2009). In the present study we mapped the active domain within statherin responsible for this effect. For this purpose statherin was truncated systematically from the N-terminal and the C-terminal side and synthetic fragments were tested for hyphae-to-yeast induction.

When N-terminal residues were deleted hyphae-to-yeast inducing activity increased with StN10-43 being the most active peptide. Further deletion of 4 N-terminal residues, in StN14-43, abolished activity. When StN10-43 was truncated from the C-terminal side hyphae-to-yeast inducing activity gradually decreased and disappeared in StN10-19. Elongation of this peptide, either N-terminally (StN6-18) or C-terminally (StN10-29 or StN10-19/37-43) restored somewhat hyphae inhibiting activity, suggesting a minimal size was required for hyphae-to-yeast induction.

Hyphae-to-yeast inducing peptides caused the detachment of firmly attached hyphae clusters from the polystyrene surface. However, the results for StN6-18, which induced yeast growth but did not cause hyphae to detach, and StN10-19 and histatin, who did the opposite, suggest two independent mechanisms.

In Chapter 4 we found that StN6-43 binds stronger to *C. albicans* than the whole statherin molecule, possibly because the absence of the negatively charged N-terminus results in less repulsion at the negatively charged *C. albicans* cell surface. Compared to StN6-43, StN10-43 shows the highest yeast growth inducing activity and binds equally well to *C. albicans* hyphae and yeasts suggesting that further truncation might be possible.

During granule maturation *in vivo* in parotid and submandibular glands, statherin can be cleaved by endo-peptidases which may give rise to the secretion of peptides StN14-43 and StN10-43 into the oral cavity. The latter peptide shows high hyphae-to-yeast-inducing activity (Messana *et al.*, 2008).

Since several statherin peptides cause transition to yeast it might be interesting to see whether the virulence is also influenced by these peptides. In a study done by Carlisle *et al.* (2009) *C. albicans* filament formation was linked to its virulence in a mouse model for systemic *C. albicans* infection (Banerjee *et al.*, 2008; Carlisle *et al.*, 2009). In this context it might be interesting to study the

effect on the expression of genes in *C. albicans* that induce hyphae formation, such as UME6 (Carlisle *et al.*, 2009).

In conclusion, we show here that truncated statherin variants show higher yeast growth induction than the full statherin molecule with the highest activity in StN10-43. Furthermore, we demonstrate that peptides that induce hyphae-to-yeast transition generally also cause hyphae to detach from the microplate wells.

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CHAPTER 6

The bacteria binding glycoprotein salivary agglutinin (SAG/gp340) activates complement via the lectin pathway

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Abstract

Salivary agglutinin (SAG), also known as gp-340 and Deleted in Malignant Brain Tumours 1, is a glycoprotein that is present in tears, lung fluid and mucosal surfaces along the gastrointestinal tract. It is encoded by the *Deleted in Malignant Brain Tumours 1 gene,* a member of the Scavenger Receptor Cysteine Rich group B protein superfamily. SAG aggregates bacteria thus promoting their clearance from the oral cavity and activates the complement system. Complement proteins may enter the oral cavity in case of serum leakage, which occurs after mucosal damage. The purpose of this study was to investigate the mode of complement activation. We showed a dose-dependent C4 deposition on SAG-coated microplates showing that either the classical or lectin pathway of complement was activated. Antibodies against mannose binding lectin inhibited C4 deposition and SAG induced no C4 deposition in MBL deficient sera showing SAG activated complement through the mannose binding lectin (MBL) pathway. Periodate treatment of SAG abolished MBL pathway activation consistent with an involvement of SAG glycans in complement activation. This provides the first evidence for a role of SAG in complement activation through the MBL pathway and suggests a potential role of SAG as a complement activating factor at the mucosal epithelia.

Introduction

Salivary agglutinin (SAG) is a glycosylated protein known for its ability to bind and aggregate a wide spectrum of micro-organisms including bacteria, fungi and viruses. SAG is encoded by the *Deleted in Malignant Brain Tumours 1 (DMBT1)* gene which is expressed by epithelial cells of the alimentary and respiratory tract and is secreted by salivary and lacrimal glands (Holmskov et al., 1997; Mollenhauer et al., 2001; Ligtenberg et al., 2007). SAG belongs to the scavenger receptor cysteine-rich superfamily, a group of proteins that are primarily involved in innate defence processes (Kang and Reid, 2003; Ligtenberg et al., 2007).

In addition to its interaction with micro-organisms, SAG stimulates alveolar macrophage migration and suppresses neutrophil oxidative burst, which are different ways to protect against micro-organisms and/or excessive tissue damage (Holmskov et al., 1999; White et al., 2005). These interactions suggest an important role for SAG as an innate immune factor.

The complement system is an important part of the humoral innate immune system (Carroll, 2008) consisting of more than 30 proteins in plasma and on cell membranes. The main effector functions of complement activation are opsonisation to facilitate phagocytosis of viral, bacterial and fungal pathogens, attraction and activation of phagocytes to sites of infection, and removal of immune complexes and apoptotic cells. Activation of the complement system can occur via three pathways, which are initiated via separate mechanisms and eventually converge in a common terminal pathway. The classical pathway is initiated by binding of its recognition molecule C1q to IgG- or IgM containing immune complexes or other repetitive structures such as the acute phase protein CRP. Subsequently, the associated serine proteases cleave and thereby activate C2 and C4 molecules. The lectin pathway essentially uses the same molecules as the classical pathway e.g. C4 and C2, except that its recognition molecules are mannose binding lectin MBL or ficolins that bind to a wide array of carbohydrate structures on pathogenic surfaces and are associated with the serine proteases MASP-1, MASP-2, MASP-3 and a truncated form of MASP-2 called Map-19. Finally, activating structures of the alternative pathway are present on the cell walls of bacteria, viruses and yeasts, whereas IgA-containing immune complexes can also activate the alternative pathway.

Although separated by an epithelial layer, contact between saliva and blood plasma is quite common in the oral cavity; for instance in periodontal diseases and after mechanical damage such as surgery or tooth brushing. Under these

circumstances blood or plasma leaks into the oral cavity, which results in mixture with saliva (Negut et al., 2007). Both saliva and blood are carriers of antimicrobial factors such as SAG and complement respectively and it has been suggested that upon mixing with saliva, the complement system may contribute to host defence mechanisms within the oral cavity (Boackle, 1991). Early research by Boackle et. al. (1993) demonstrated the potential of human SAG to bind to C1q and to activate C1.

The aim of the present study was to investigate the activation of complement by SAG in detail. We observed that *in vitro* SAG activates the complement system primarily via the MBL mediated lectin pathway and that fucose on SAG is a critical determinant of MBL binding and complement activation.

Materials and methods

Serum samples

Serum was collected from healthy volunteers. After drawing blood, it was left to coagulate for 1 hour at room temperature (RT) after which, the blood clot was removed from the serum. Serum was stored at -80˚C until testing.

Antibodies

Monoclonal anti-C4-10 against C4 and CLB-MBL/1 against MBL were described previously ((Wolbink et al., 1993; Bultink et al., 2006). Anti-MBL/1 inhibits binding of MBL to its substrates. Monoclonal anti-C1q-85, which interferes in the interaction of C1q with its ligands and therefore inhibits classical pathway activation, has
been described before (Hoekzema et al., 1988; McGrath et al., 2006). Monoclonal antibody 213-06 was obtained from the antibodyshop (Gentofte, Denmark). Biotinylated Ulex europaeus agglutinin-1 (UEA-1), specific for terminal fucose, was from Pierce (Rockford Il, USA). Digoxigenin labeled Datura stramonium agglutinin (DSA), specific for terminal N-acetylglucosamine, was obtained from Roche
(Roche Diagnostics GmbH, Mannheim, Germany). Galanthus nivalis agglutinin (GNA), specific for terminal mannose, was obtained from Roche. Aggregated human IgG (AHG) was prepared by incubating purified human IgG at a concentration of 20 mg/ml for 20 min at 63 °C.

Collection of crude SAG and purification of SAG

SAG was purified by affinity adsorption of parotid saliva to *S. mutans* as described previously (Ligtenberg et al., 2000). *S. mutans UA159* was cultured in 100 ml Todd Hewitt Broth by overnight (o/n) incubation at 37 °C. The bacteria were pelleted by centrifugation at 15,000 x *g* at RT, washed 2 times in phosphate buffered saline (PBS), pH 7.4 and then resuspended in 5 ml PBS. 35 ml of parotid saliva was collected with a Lashley cup under stimulation with menthol chewing gum and centrifuged for 2 minutes to get rid of debris. The bacteria suspension was then mixed with parotid saliva and incubated by shaking for 1 hour at 37 °C. The bacteria were then pelleted by centrifugation (15,000 x *g*, RT) and washed 2 times with PBS containing 1 mM $CaCl_2$. Bacteria were resuspended in 4 ml PBS containing 10 mM EDTA to elute the bound SAG and incubated for 20 minutes by shaking at RT. Bacteria were then

pelleted and discarded. The supernatant was then again centrifuged for 10 min at 20,000 x *g* at RT after which the pellet was discarded and the supernatant was transferred to a separate tube. The purity of the SAG preparation was tested by means of SDS-PAGE and Coomassie staining (Fig. 1) showing one major band at approximately 340 kD. SAG concentration was determined by Bio-Rad protein microassay procedure for microtiterplates and ranged from 70 to 80 µg/ml.

Periodic acid treatment

For periodic acid oxidation of SAG carbohydrates, SAG was first diluted in coating buffer (0.1 M carbonate buffer, pH 9.6) and immobilized on 96-wells plates (Nunc *MaxiSorp*™, Roskilde, Denmark*)* by o/n incubation at 4 °C. The SAG coated plates were then incubated for 1 hour in 0.1 M in acetic acid (pH 4.2) containing 20 mM $NaIO_4$. The plates were then washed 3 times with H_2O followed by a 1 hour reduction with 50 mM $NaBH_4$ in PBS. The plates were washed then 3 times with H_2O and used for testing.

Functional MBL concentration

Functional MBL concentration was determined in a solid phase ELISA with mannan coated onto the solid phase and detection with biotinylated monoclonal antibody anti-MBL/1. Briefly, microtiterplates (Nunc Maxisorp) were coated o/n at RT with mannan (Sigma-Aldrich, St Louis, MO, USA; 50 µg/ml in 0.1M carbonate buffer, pH 9.6). In between incubations microtiterplates were washed five times with H_2O. Sera were diluted in 20 mM Tris (pH 7.4) supplemented with 150 mM NaCl, 10 mM $CaCl_2$, 0.02% Tween and 0.2% gelatin (TTG/Ca) and incubated for 1 hour at RT. Bound MBL was detected by biotinylated anti-MBL/1 (2 µg/ml in TTG/Ca) for 1 hour at RT. Functional MBL concentration was calculated on a titration curve of a serum pool of healthy donors. MBL concentration below 500 ng/ml was considered MBL-deficient.

C4 deposition on SAG

Microtiterplates (Nunc MaxiSorp) were coated o/n at 4 °C with 10 µg/ml purified SAG (either untreated or treated with periodic acid) in 0.1M carbonate buffer, pH 9.6. For control experiments microtiterplates were coated o/n at RT with 20 µg/ml AHG in PBS or 50 µg/ml mannan in 0.1M carbonate buffer. All incubations were performed in a final volume of 100 µl. Serum samples were diluted 1:10, followed by threefold dilutions in Veronal buffered saline, pH 7.4 supplemented with 10 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.02% Tween-20 and

incubated for 1 hour at RT. To specifically inhibit either the classical or the lectin pathway of complement, all serum dilutions were incubated in the presence of blocking monoclonal antibodies against C1q (anti-C1q-85, 50 µg/ml) or MBL (anti-MBL/1, 10 µg/ml). Subsequently, plates were washed five times with PBS/0.02% Tween-20. To detect C4 deposition, plates were incubated for 1 hour at RT with biotinylated anti-C4-10 (0.25 µg/ml), diluted in high performance ELISA buffer (HPE; Business Unit reagents, Sanquin, Amsterdam, the Netherlands). After five washes with PBS/0.02% Tween-20, plates were incubated with streptavidin-peroxidase (Amersham/Pharmacia, Uppsala, Sweden) (1:1000 in HPE) for 30 minutes at RT. After five washes with PBS/0.02% Tween-20, the ELISA was developed with 100 µg/ml tetramethylbenzidine in 0.11 M sodium acetate (pH 5.5) containing 0.003% v/v H_2O_2. Substrate conversion was stopped by addition of 100 µl H_2SO_4. Absorbance was measured at 450 nm with a Titertek multiscan.

MBL binding to SAG

MBL binding to SAG (either untreated or treated with periodic acid) was detected in an ELISA similar to the C4 deposition ELISA with some modifications. In short, plates were coated with SAG as described above. Serum samples were diluted 1:3 in TTG/Ca followed by threefold dilutions and incubated at RT. MBL binding was detected with biotinylated anti-MBL/1 (2 µg/ml in TTG/Ca). ELISA was developed as described above.

Detection of carbohydrates on SAG

SAG was coated *in* twofold serial dilutions (0-20 µg/ml in 0.1M carbonate buffer, pH 9.6) o/n at 4 °C in 96 wells microtiterplates (Nunc *MaxiSorp). After washing,* detection of carbohydrates was performed using digoxigenin (DIG)-labeled lectins GNA and DSA for the detection of mannose and N-acetyl-D-glucosamine (GlcNAc) respectively, and biotinylated UEA-1 lectin for the detection of fucose. As a control for coating of SAG we used monoclonal antibody HYB 213-06 that recognizes part of the protein core SAG (Bikker et al., 2002). Lectins and antibody were diluted 1:2000 (final concentration 0.5 µg/ml) in Tris Buffered Saline (TBS) at pH 7.5 containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ supplemented with 2% BSA and 0.1% Tween-20 and incubated for 1 hour. The plates were then washed 3 times with TBS/0.01% Tween-20 and rinsed with H_2O. After washing, DIG-labeled lectins were incubated with 75 mU/ml peroxidase-conjugated anti-DIG Fab-fragments (Boehringer Mannheim, Roche, Netherlands). Biotinylated UEA-1 was

incubated with streptavidin-peroxidase (Amersham/Pharmacia). HYB 213-06 was incubated with 0.65 µg/ml peroxidase-conjugated rabbit-anti mouse antibody (Dako, Belgium). All incubations were done for 1 hour in TBS containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ supplemented with 2% BSA and 0.1% Tween-20 and then were washed 3 times with TBS/0.01% Tween-20 and rinsed with H_2O. Enzymatic-staining was done using SIGMA*FAST*™ OPD tablet-kit (Sigma-Aldrich, Netherlands). The reaction was stopped by adding 50 µl 0.05 M sulfuric acid. Microtiterplates were read at 450nm using a MR7000 ELISA-reader (Dynatech, VA, USA).

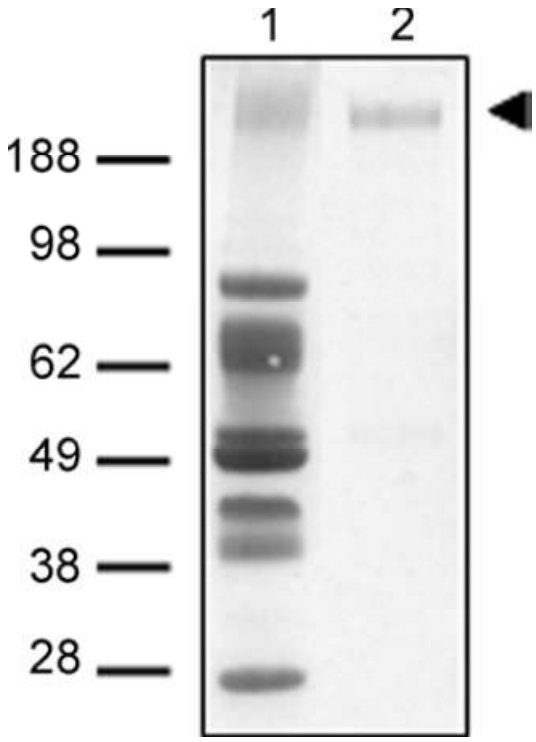

Fig 1. SDS-PAGE analysis of purified SAG. SAG was purified by affinity adsorption of parotid saliva to *S. mutans*. Purity of SAG was shown by SDS-PAGE and Coomassie staining. Lane (1). Parotid saliva, Lane (2) SAG extract. The location of SAG is indicated by the arrowhead.

Results

Complement activation by SAG

Purified SAG (Fig. 1) was immobilized on microtiterplates in twofold serial dilutions (0 - 20 µg/ml) and tested for complement activation by detection of C4 deposition as described (Fig. 2). Serum of three healthy subjects (diluted 1: 50) clearly demonstrated C4 deposition on immobilized SAG indicating that SAG is able to activate the complement system. C4 deposition was dose dependent and was completely absent in the presence of 10 mM EDTA, ruling out non-specific direct binding of C4 from the serum to the coat.

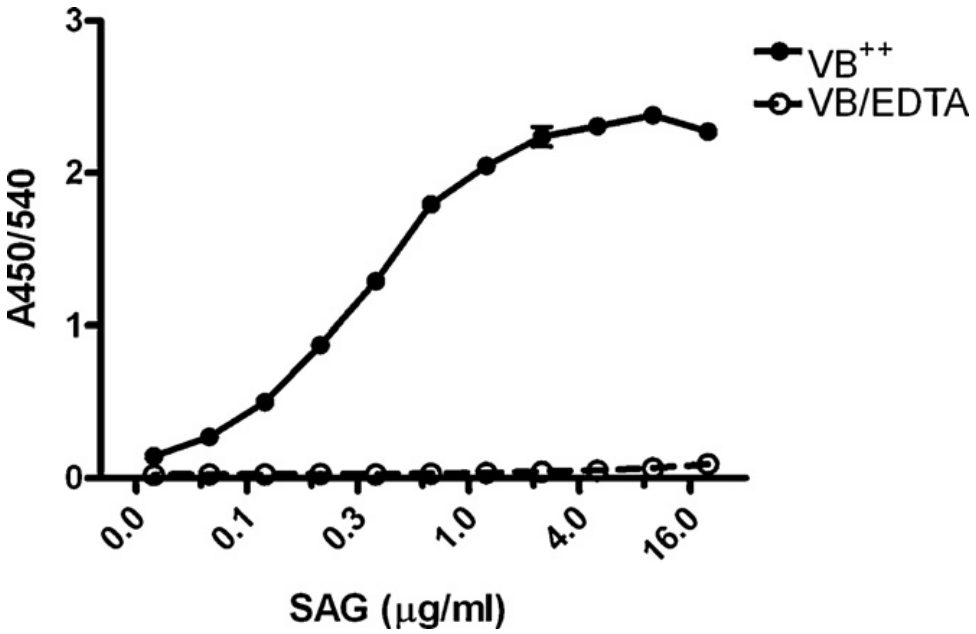

Fig 2. Immobilized SAG induces C4 deposition. Serum was incubated (1:50 dilution) on serially diluted immobilized SAG in VB^{++} or in VB/10 mM EDTA. Results represent mean and SEM (error bars) of 3 MBL-sufficient donors.

C4 activation by SAG dependent on MBL

C4 activation may be the result of either the classical or the lectin complement pathway. To identify which complement pathway is activated by SAG, either the classical or the lectin pathway was inhibited by incubation with blocking monoclonal antibodies against C1q and MBL (Fig. 3). Incubation with anti-C1q-85 and anti-MBL/1 completely blocked C4 deposition on AHG and mannan, respectively (Supplementary figure 1).

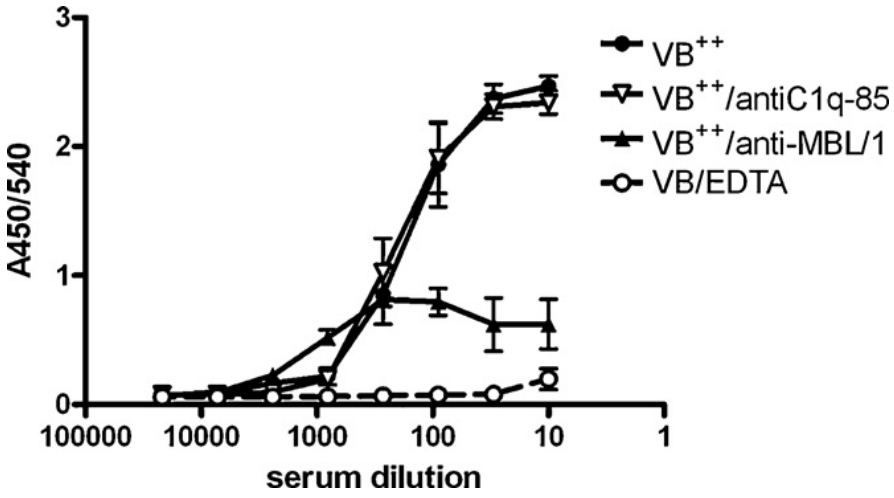

Fig 3. C4 deposition on immobilized SAG is primarily mediated by MBL
Serum was incubated on immobilized SAG in VB^{++} in the presence of inhibiting monoclonal antibodies anti-C1q-85 and anti-MBL/1. Anti-MBL/1 significantly reduced C4 deposition, whereas anti-C1q-85 only had a minor effect. Results represent mean and SEM (error bars) of 3 MBL-sufficient donors.

Unexpectedly, blocking of C1q caused only mild decrease in complement activation by SAG, (Fig. 3). Incubation with anti-MBL/1, however, blocked most C4 deposition on SAG, indicating that MBL plays the most important role in SAG induced complement activation.
To further investigate whether MBL is responsible for C4 deposition on SAG we compared three MBL-sufficient sera with three MBL-deficient sera in parallel for complement activation by immobilized SAG. MBL levels of healthy volunteers were measured by ELISA and three sera with undetectable levels of MBL were selected for further experiments. Incubation with MBL-deficient sera resulted in drastically lower C4 deposition as compared with the MBL-sufficient sera (Fig. 4A). To ensure that complement activation was intact in the MBL-deficient sera, a C4 deposition assay was performed on immobilized AHG. C4 activation on AHG was completely normal in all tested sera (Fig. 4B), indicating that the observed differences between MBL-sufficient and -deficient sera could not be attributed to different functional C4 levels.

Fucosylation of SAG is critical for complement activation
Since SAG is a highly glycosylated protein with 25-40% of its molecular weight made up of carbohydrates it is very likely that, being a lectin, MBL binds to certain sugar residues on SAG. Three MBL sufficient sera were tested for C4

deposition on both untreated SAG and periodic acid treated SAG. As depicted in figure 5, both C4 deposition and MBL binding to SAG were strongly reduced after periodic acid treatment of SAG, indicating that the carbohydrates on SAG are responsible for complement activation, rather than the polypeptide part of the molecule. MBL is known to bind mannose, β-GlcNAc and α-L-fucose containing carbohydrate moieties (Turner, 2003; Keshi et al., 2006). We investigated the presence of these carbohydrates on SAG-coated microplates with carbohydrate specific lectins (Fig. 6). We could detect fucose with UEA-1 lectin, but we detected no GlcNAc with DSA lectin or mannose with GNA lectin. After periodic acid treatment fucose was not detected anymore. GlcNAc became detectable after periodic acid treatment, but mannose was still undetectable. Periodic acid did not affect SAG coating as shown with HYB 213-06 antibody, which recognizes a peptide fraction of salivary agglutinin (Fig. 6). These results show that complement activation corresponds with the availability of fucose on SAG.

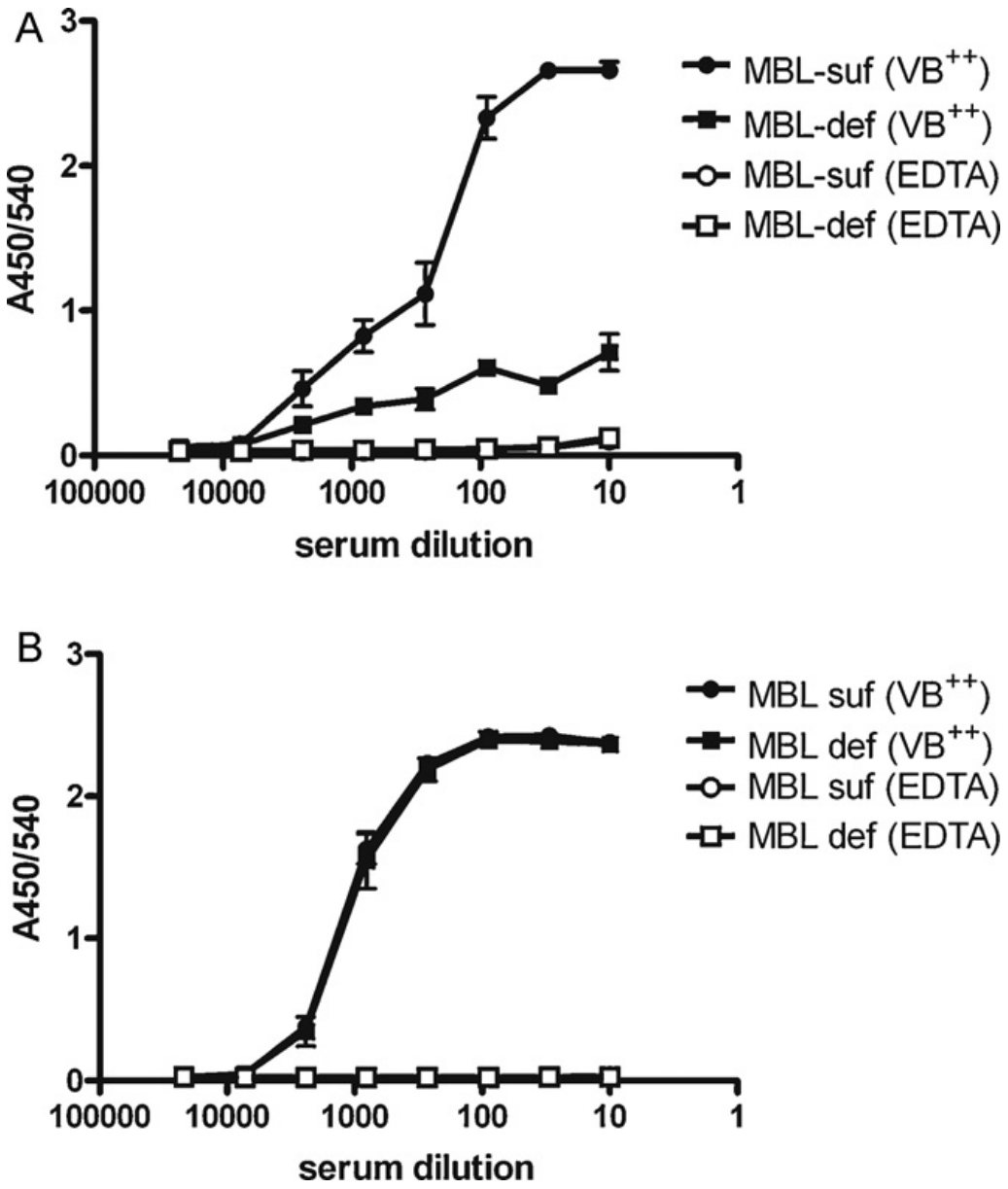

Fig 4. C4 deposition on SAG in MBL-sufficient vs MBL-deficient sera.
Serum obtained from both MBL-sufficient and MBL-deficient donors was incubated on (A) immobilized SAG or (B) immobilized AHG in VB^{++} or in VB/10 mM EDTA. Results represent mean and SEM (error bars) of 3 MBL-sufficient donors and 3 MBL-deficient donors.

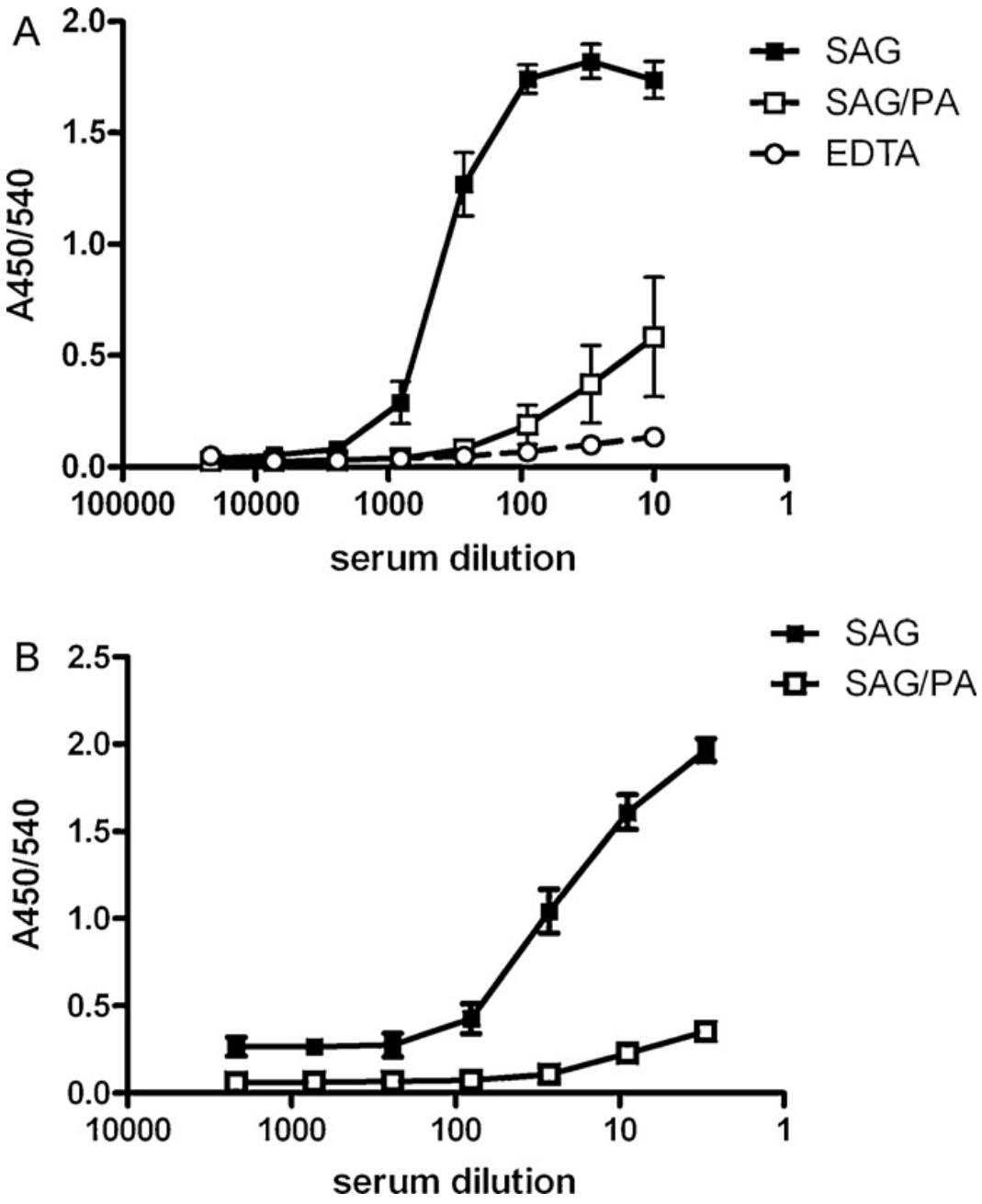

Fig 5. Periodic treatment of SAG abolishes C4 deposition and MBL binding
Immobilized SAG was treated with periodic acid (SAG/PA) or left untreated. Hereafter, C4 deposition (A) was tested with serum diluted in Veronal buffer with Ca^{2+} and Mg^{2+} (VB^{++}) or in Veronal buffer with 10 mM EDTA. In addition, MBL binding was detected on either untreated SAG or SAG/PA (B). Results represent mean and SEM (error bars) of 3 MBL-sufficient sera.

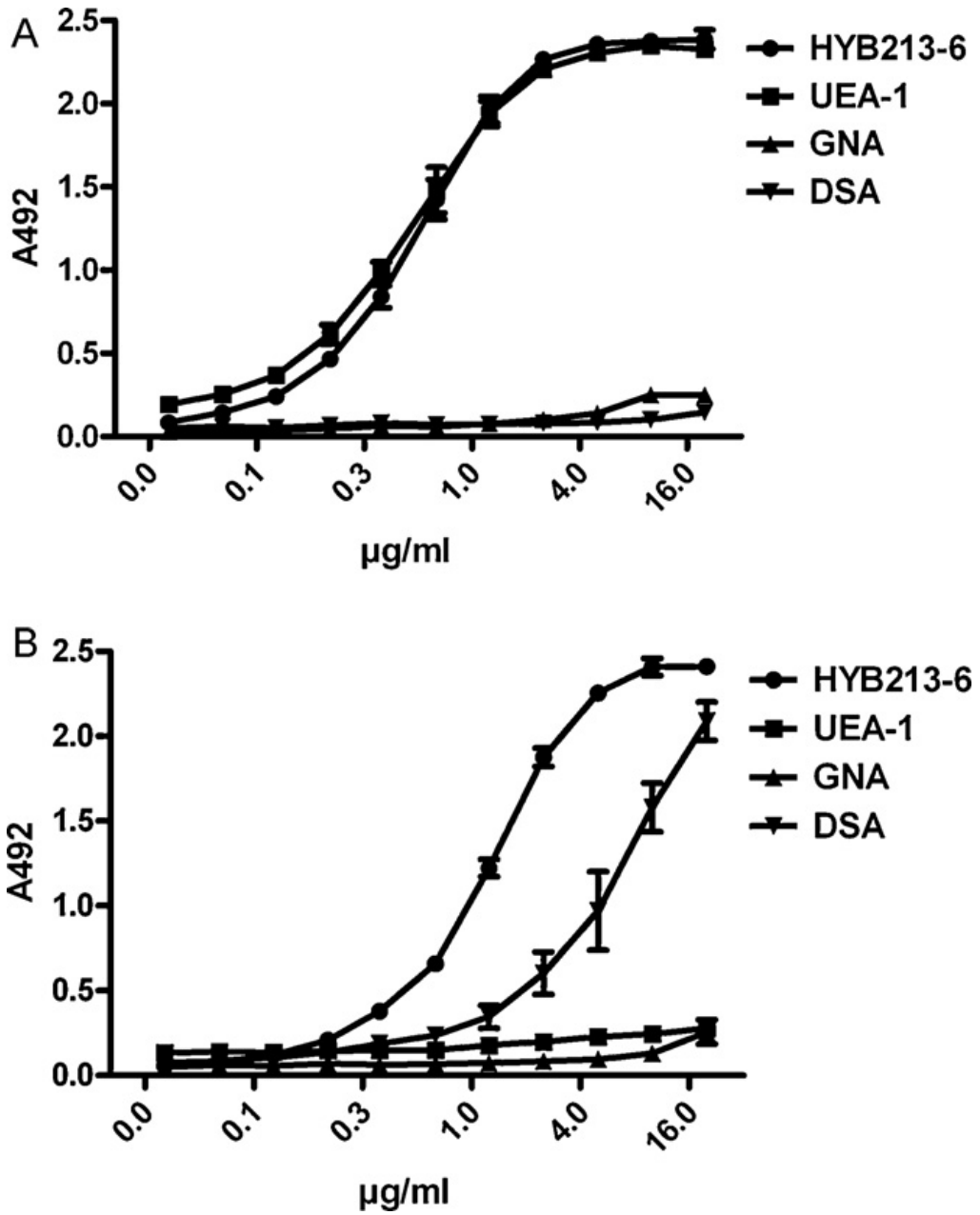

Fig 6. Detection of sugars on SAG, by lectin binding, after periodic acid treatment
Immobilized SAG was either left untreated (A) or treated with periodic acid (B). HYB213-6 antibody, raised against a peptide part of SAG, was used as a control for coating. Fucose was detected using UEA-1 lectin, GlcNAc was detected using DSA lectin and mannose detection was done using GNA lectin. Results represent mean and SD (error bars) of three samples. Of the potential MBL binding carbohydrates only fucose was available on untreated SAG. After treatment with periodate GlcNAc could by detected.

Discussion

The present study confirms that SAG is able to activate the complement system as has been described before (Boackle et al., 1993). However, in this study we demonstrate that complement activation by SAG is primarily mediated by the lectin pathway and to a much lesser extent by the classical pathway. Although the latter had already been reported before (Boackle et al., 1993), the potent activation of the lectin pathway by SAG has not and may be relevant with respect to the physiological role of SAG at the mucosal lining. Moreover, the activation of the classical pathway has only been shown by the appearance of C1r-C1-inhibitor complexes on Western blot, which is not a quantitative assay. The observed classical pathway activation may thus be of minor importance compared to the lectin pathway.

In our hands, blockage of C1q by use of an inhibiting anti-C1q monoclonal antibody showed a slight inhibiting effect on C4 deposition on SAG-coated plates which may explain the appearance of C1r-C1 complexes as described by Boackle *et al.* (1993)
In contrast, blocking MBL binding with a monoclonal antibody, most C4 deposition on SAG could be prevented indicating that the lectin pathway plays an important role in SAG mediated complement activation. This was confirmed by the incubation of MBL-deficient sera on immobilized SAG which resulted in very low C4 deposition.
The remaining C4 activation that was observed in MBL-deficient sera, could not be inhibited by blocking the classical pathway of complement. In addition, co-incubation of anti-MBL and anti-C1q monoclonal antibodies never completely blocked C4 activation, indicating that another mechanism of C4 activation exists on SAG. One possible explanation for the observed C4 deposition in absence of MBL is ficolin mediated complement activation. Ficolins are recognition molecules that are very similar to MBL and also associated with MASPs, but recognizing different carbohydrate structures. Considering the heterogeneity of carbohydrates on SAG there might well be structures that are recognized by ficolins (Issa et al.).
Next to mannose-containing carbohydrate structures MBL also recognizes GlcNAc- and fucose-containing glycans (Turner, 2003; Keshi et al., 2006). Complement activation by immobilized SAG, corresponded with the presence of fucose-residues, since after periodic acid treatment fucose was no longer detectable and both MBL binding and C4 deposition disappeared. The

carbohydrate chains on SAG are heavily fucosylated and contain blood group related structures like Lewis$^{a/x}$ and Lewis$^{b/y}$ antigens (Issa et al.). It will be interesting to investigate whether differences between individuals in glycosylation of SAG may result in differences in complement activation.
MBL levels vary widely in the normal population and approximately 25% of the people in the Western world have strongly decreased functional MBL (Garred et al., 2003). A number of studies have shown that individuals with low levels of MBL have an increased risk for infections, particularly when immunity is already compromised: for example in infants, patients with cystic fibrosis, after chemotherapy and transplantation (Turner, 2003). It is tempting to speculate about a potential synergistic antimicrobial effect between SAG and MBL-mediated complement activation. Therefore, it would be interesting to investigate the prevalence of oral infections in relation to MBL levels. In addition, the effect of SAG on MBL-mediated opsonophagocytosis on yeasts and bacteria deserves further research.
Although saliva is secreted in the oral cavity and serum is present inside the blood vessels interaction frequently occurs. In case of gingivitis, periodontal disease, but also after tooth brushing, serous exudates enter the oral cavity and mix with saliva (Henskens et al., 1996; Hoek et al., 2002; Giannobile et al., 2009). Many salivary diagnostic tests, such as for HIV, are based on the presence of serum components in saliva (Malamud and Wahl). Serous exudates contain functional complement proteins that may interact with salivary proteins to potentiate their antimicrobial action.
One question remains open whether complement activation by SAG is only beneficial to the host or can be detrimental as well. On the one hand, if SAG and MBL work synergistically leading to better phagocytosis of bacteria this could strengthen the host defence against oral infections. On the other hand, if complement activation by SAG leads to production of more pro-inflammatory complement split products such as C3a and C5a, this might eventually lead to increased influx of neutrophils thereby increasing inflammation in the oral cavity. The release of neutrophilic granule contents such as elastase or reactive oxygen species may induce more unwanted tissue damage giving more clinical problems. In periodontal disease, that is characterized by serum leakage in the mouth, reactive oxygen species have been described to play a role (Hu et al., 2009).
In conclusion, the present study demonstrates that the antimicrobial salivary protein SAG is capable of activating complement via the lectin pathway. The functional relevance of complement activation by SAG at the mucosal surfaces

remains to be investigated. The recently reported SAG-deficient mice (Madsen et al.; De Lisle et al., 2008) may be of help in this context.

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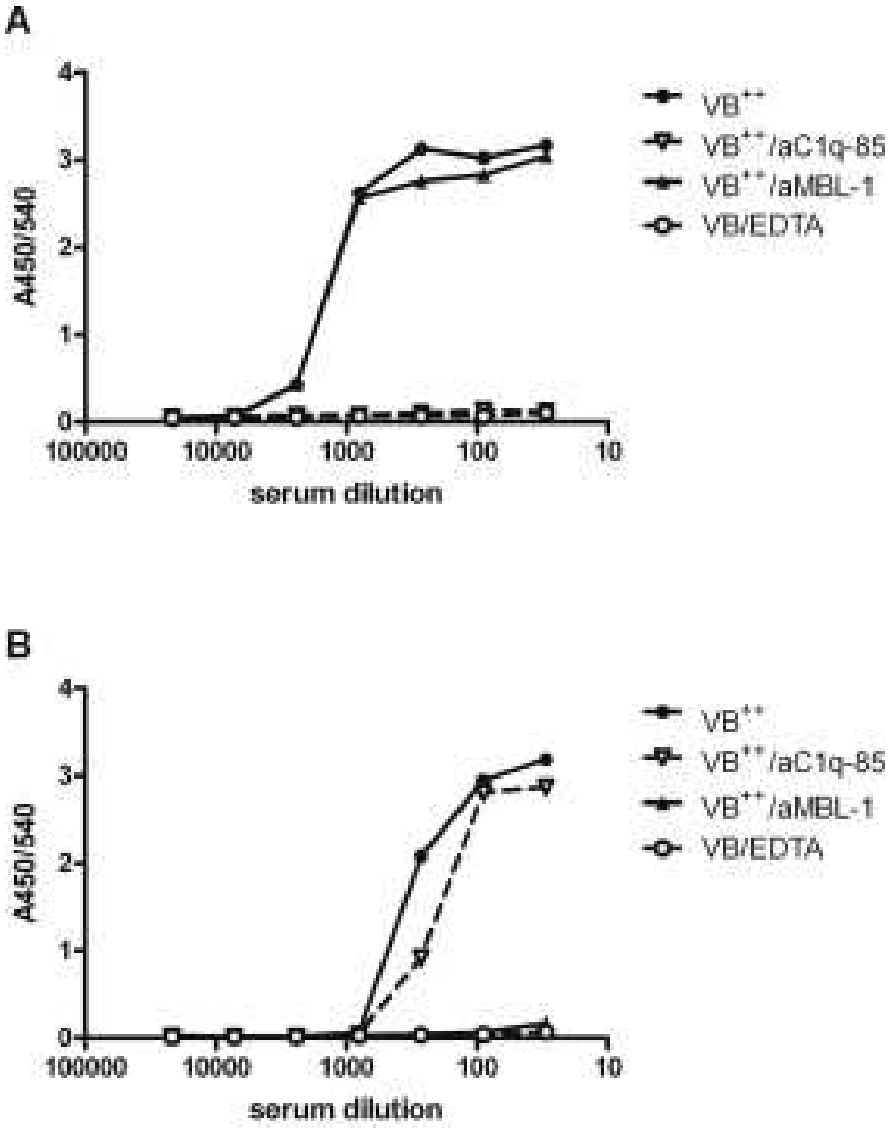

Supplementary to Figure 1. Complement activation through AHG (A) or mannan (B).
Microtiterplates were coated with AHG (A) or mannan (B) and incubated with serial dilutions of serum in VB^{++} buffer in the presence of anti-C1q-85 or anti-MBL/1. Anti-C1q-85 inhibited C4 deposition on AHG and anti-MBL/1 inhibited C4 deposition on mannan.

Chapter 7

General Discussion

[29]Alleen, zie toch: ik heb ontdekt, dat God de mensen recht gemaakt heeft, maar zij zoeken vele bedenkselen.
Prediker 7:29

General Discussion

The human oral cavity is inhabited by a wide variety of micro-organisms. One of the factors that play a dominant role in the maintenance of a healthy oral ecosystem is saliva. The crucial role of saliva becomes apparent when saliva secretion is impaired, e.g. in patients with the Sjögren's syndrome, after radiation therapy, surgical removal of diseased salivary glands or as side effect of medication. Under these conditions a shift in the oral microflora may develop, resulting in the outgrowth of opportunistic pathogens, e.g. *Candida albicans*. Saliva contains numerous antimicrobial proteins and peptides that protect the oral tissue against microbial invasion and infection. These include proteins with bacteriostatic, bactericidal and fungicidal activity such as lysozyme, lactoferrin, cystatins and histatins (Nieuw Amerongen and Veerman, 2002; Tenovuo, 2002; Nieuw Amerongen *et al.*, 2004; Ligtenberg *et al.*, 2007). Other proteins, such as statherin, adsorb onto dental surfaces. By functioning as receptors for specific microbial adhesions they modulate the composition of the microflora on the tooth surface. Salivary proteins in solution, including immunoglobulins, particularly secretory-IgA, MUC7 and salivary agglutinin (SAG) bind and agglutinate oral bacteria promoting their removal from the oral cavity (Rossi *et al.*, 2003; Ligtenberg *et al.*, 2007; Kesimer *et al.*, 2009). In this thesis especially the biological activity of two salivary components has been studied: salivary agglutinin (SAG) and statherin.

SAG was originally discovered as a salivary component mediating agglutination of oral streptococci, in particular *S. mutans* (Ericson and Rundegren, 1983). Due to its ability to bind and agglutinate this cariogenic bacterium, SAG has been considered to play an important role in the protection against dental caries (Ericson and Rundegren, 1983; Carlen *et al.*, 1998). SAG was characterized biochemically as a 300-400 kDa glycoprotein consisting for 25 % of its molecular mass of carbohydrate residues. During years SAG was thought being specifically for saliva, particularly parotid saliva. However, in later studies it was proven that SAG, lung glycoprotein340 (gp340) and Deleted in Malignant Brain Tumours 1 (DMBT1) are all encoded by one and the same gene, namely the *DMBT1* gene on chromosome 10. These proteins have identical protein sequences, but may be different in glycosylation. SAG is a member of the scavenger receptor cysteine-rich superfamily (SRCR) of proteins, a superfamily of surface-bound or secreted proteins typically possessing SRCR domains. Members of this group are usually involved in innate immune processes (Sarrias *et al.*, 2004). SRCR domains consist of 110 amino acids and

their tertiary structure is stabilized by 3 or 4 disulfide bridges. SRCR domains are well conserved within all metazoa and usually are involved in ligand binding, as is the case with SAG. Bikker *et al.* (Bikker *et al.*, 2002) demonstrated that the binding site for bacteria is located in the SRCR domains. The high homology of the SRCR domains in SAG justified the design of a consensus sequence. Based on this consensus sequence synthetic peptides were designed and tested for binding to bacteria. A 16-mer amino acid sequence (**QGRVEVLYRGSWGTVC**), derived from the SRCR domains of SAG (SRCR-P2), bound to a wide variety of micro-organisms. In **Chapter 2** we showed that there is a good correlation between the binding of SAG and the SRCRP2 peptide to a wide variety of microbial species supporting the concept that the sequence encompassed by the SRCRP2 peptide represents the major bacteria-binding site of SAG. By truncation of the peptide combined with an alanine scan, Bikker *et al.* (Bikker *et al.*, 2004) revealed an 11-mer peptide in which 6 amino acids were essential for binding of *S. mutans* (Q**G**R**VEVL**YRGS**W**GTVC). We investigated in more detail the sequence that allows this peptide to bind to such a wide variety of bacterial species. We found that different residues are involved depending on the bacterium. In all cases are valine residues at position 4 and 6, tyrosine at position 8 and tryptophan at position 12 essential for binding. Amino acids that were often, but not always, involved in binding were arginine at position 3, glutamic acid at position 5, leucine at position 7 and serine at position 11. Of the SRCR-P2 residues that are always essential for binding, three out of four are highly conserved in the SRCR superfamily (Figure 1). A hidden Markov model logo of the SRCR domain shows that the valine residues at position 4 and 6, and tryptophan at position 12 were highly conserved within the SRCR superfamily, supporting the idea that these residues are functionally important. Also the bacteria-binding domain of two other SRCR proteins, Sp-α and CD 163, is homologous to SRCR-P2 fragment (Sarrias *et al.*, 2005; Fabriek *et al.*, 2009).

The broad binding spectrum of SAG is typical for so called pattern recognition proteins. These are molecules that bind to conserved molecules on pathogenic micro-organisms, called pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide, lipoteichoic acid and peptidoglycan. SAG was indeed shown to bind to LTA and phosphorylated groups on LPS (End *et al.*, 2009). In **Chapter 3** we show that SAG not only recognizes bacterial components, but also binds to *C. albicans.* Adhesion experiments in the presence of mannan showed no inhibition, suggesting that mannan is not the

PAMP recognized by SAG on the yeast surface. Future research should characterize the molecular binding between *C. albicans* and SAG.

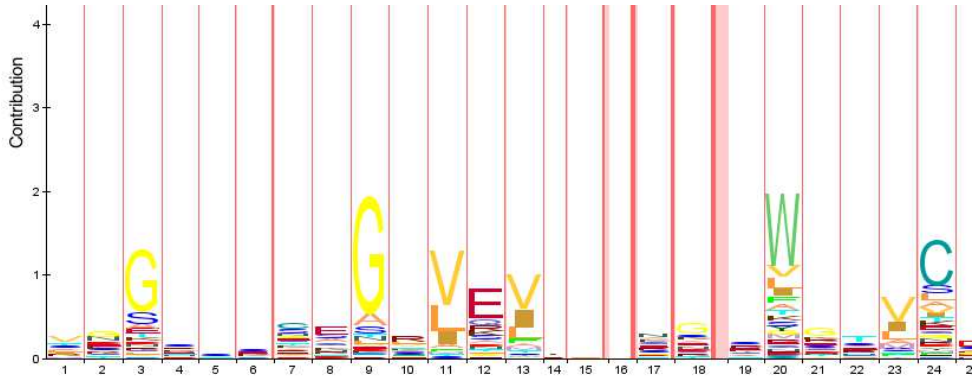

Fig. 1. Hidden Markov models logo of a fragment of the SRCR domain. HMM logos are a visual tool for showing the degree of conservation within a protein family. The height of the amino acids represents their probability to be present in the protein family compared to the background. Different colors are given for amino acids of different charge and hydrophobicity. The amino acids 8-24 correspond with the SRCR-P2 peptide. This figure shows that valine at position 11 and 13, and tryptophan at position 20 are well conserved. Within the SRCR-P2 peptide these amino acids were also essential for binding to bacteria.

There is a number of differences between bacteria binding and *C. albicans* binding of SAG. Binding of SAG to bacteria is abolished after chemical reduction of SAG. In contrast, the SAG-*C. albicans* interaction was not affected by chemical reduction. Another difference compared to bacteria binding is that adhesion of *C. albicans* to SAG coated surfaces is independent of Ca^{2+}-ions. The binding of *C. albicans* to SAG shared some characteristics with that of binding to influenza virus, that binds to sialic acid residues on SAG. This binding is also independent of Ca^{2+}-ions and not affected by chemical reduction (Hartshorn *et al.*, 2003).

In **Chapters 4 and 5** the effect of saliva on hyphae formation of *C. albicans* was studied. *C. albicans* is a multimorphic fungus growing in yeast form or hyphae form. Hyphae are more adhesive and more virulent than yeasts, the predominant form in the oral cavity. Ogasawara (Ogasawara *et al.*, 2007) showed that bovine salivary mucins inhibited hyphae formation of *C. albicans.* Therefore, we investigated in which way human saliva influences hyphae growth of *C. albicans*. We found that saliva induces transition to yeast growth under conditions favouring hyphae formation. Since mucin-free parotid saliva also induced yeast growth, we speculated that SAG, which is the most mucin-

like molecule in parotid saliva, might be responsible for transition to yeast growth. However, purified SAG did not show any effect on this process, prompting us to search for another parotid component promoting yeast-induction. By fractionation of parotid saliva it was shown that the salivary statherin, 43-mer peptide, was responsible for this activity. Testing of truncated variants of statherin for yeast growth induction revealed that deletion of the negatively charged N-terminal amino acids in StN6-43 enhanced the hyphae inhibiting activity. Possibly, there is repulsion between the negatively charged cell surface and the negatively charged N-terminus of statherin. This was also suggested by the finding that StN6-43 binds better to *C. albicans* than the full length statherin. The hyphae inhibiting activity was further increased by deletion of another 4 amino acids at the N-terminus (StN10-43), but in this case no further negative charges are deleted. Binding of this peptide to *C. albicans* was comparable with StN6-43.

Inhibition of hyphae formation is a new protective mechanism by which saliva may prevent *C. albicans* infection. Hyphae formation may be triggered by all kinds of different stimuli, for instance by addition of serum or N-acetylglucosamine to growth medium, or by growth at 37 ºC. These circumstances trigger the expression of genes that induce hyphae formation. It will be interesting to investigate the effect of statherin on the expression of hyphae inducing genes.

SAG and complement

SAG has been implicated in the protection of the oral tissue against bacterial colonization (Madsen *et al.*; Carlen *et al.*, 1998; Ligtenberg *et al.*, 2007; Leito *et al.*, 2008). This has generally been attributed to its binding and agglutination of a wide variety of micro-organisms. However, SAG is only a minor component in saliva, making up less than 1% of the salivary protein content. Other bacteria-binding proteins, such as S-IgA, proline-rich glycoprotein and MUC7 are much more predominant in saliva. Therefore, it is tempting to speculate that inhibition of bacterial colonization is not the only physiological function of SAG. An earlier study by Boackle *et al.* (Boackle *et al.*, 1993) suggested that SAG is also able to activate the complement system, an amplifying cascade of proteolytic cleavages of specific proteins in the blood and tissues, which cooperates with the adaptive immune system in the clearance of pathogens from the body. Under healthy conditions, little if any interaction takes place between saliva and the complement system. However, in individuals with oral infections, such as periodontal disease, serum exudates will mix with

saliva at the gingival margin, resulting in mutual interaction between salivary and serum defensive systems such as the blood coagulation system (Berckmans *et al.*) and the complement system (Boackle *et al.*, 1993). In **Chapter 6** we investigated in more detail the molecular basis underlying the SAG-mediated activation of the complement system.

Three biochemical pathways activate the complement system: the classical pathway, the lectin pathway and the alternative pathway (Figure 2).

1. The classical pathway is triggered when antibody-antigen complexes bind to C1q, a part of the C1 complex.
2. The lectin pathway is activated by binding of mannose-binding lectin (MBL) or ficolins to carbohydrate residues on the surface of micro-organisms. The classical and lectin pathway both lead to C4 activation which is not the case in the alternative pathway.
3. The alternative pathway is initiated by spontaneous hydrolysis of complement factor C3 to C3a and C3b. In the absence of a pathogen, these fragments will rejoin, resulting in deactivation. When C3b encounters a pathogen, it will bind covalently to its surface. This initiates a cascade of processes leading to further activation of C3.

All three pathways ultimately lead to activation of C3-convertase. This protease causes a further cascade of proteolytic activations, ultimately generating a number of active products that stimulate a variety of immune-related processes, such as opsonization, chemotaxis, vascular permeabilization and formation of the membrane-attack complex, the cytolytic endproduct of the complement cascade.

Boackle *et al.* (1993) showed that SAG was able to activate the complement system through the classical pathway. However, by analysis of the downstream processes we demonstrated in **Chapter 6** that SAG activates the complement system mainly via the MBL pathway. Complement activation by SAG resulted in C4 deposition and was blocked by anti-MBL antibodies, whereas anti-C1q had no effect. Furthermore, with MBL-deficient sera no SAG-mediated complement activation occurred.

Complement activation by SAG was putatively mediated by fucose-containing oligosaccharides on SAG, since removal of this carbohydrate abolished the SAG-mediated complement activation. Since fucose residues are present on other salivary glycoproteins, e.g. the salivary mucins, as well, it will be interesting to investigate to what extent these are also able to activate the

complement system. The presence and availability of fucose epitopes in on salivary glycoproteins shows large interindividual variation and depends on, among other things, the blood group and secretor status. Future research should find out whether differences in glycosylation of SAG determine activation of complement.

The effect of SAG on complement activation is only a first step in investigating the role of complement activation in the oral cavity. Future research should find out which other salivary proteins are involved in complement activation and what the effect of complement activation is on oral bacteria.

Conclusively, we can state that two novel issues of the salivary defence are addressed in this thesis.
- First, the role of SAG in the innate immunity seems not to be restricted to direct clearance of bacteria, but probably also involves activation of the complement system in serum exudate.
- Second, next to these defence mechanisms, a novel antimicrobial defence mechanism in saliva was discovered, namely the statherin-mediated inhibition of hyphae formation by the yeast *C. albicans*.

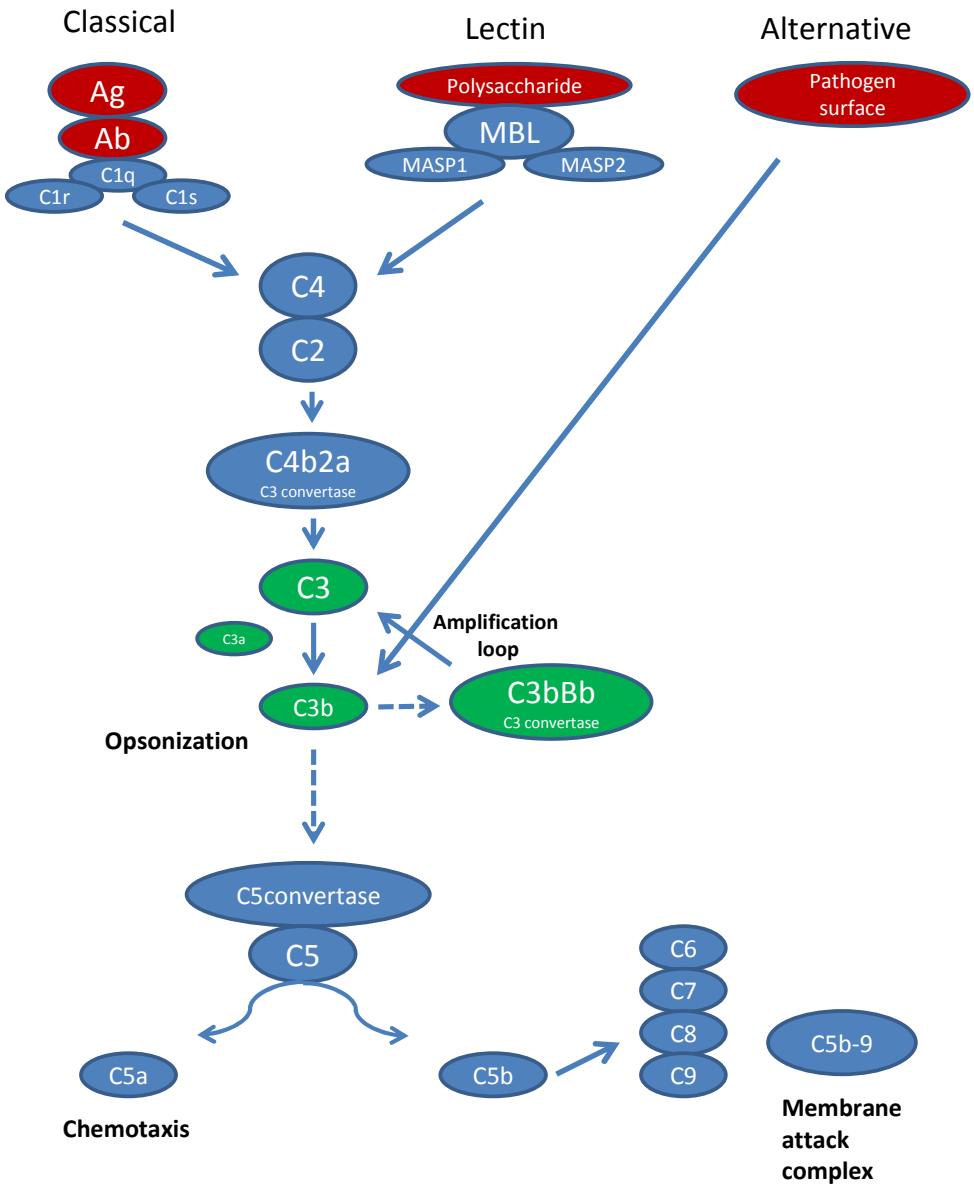

Fig. 2. The complement system. There are three pathways of complement activation, shown at the top of the figure, the classical(1), lectin (2) and alternative (3) pathway.

(1) Binding of the C1 complex initiates the classical pathway.

(2) Binding of the MBL/MASP-2 comlex initiates the lectin pathway. Both the classical and lectin pathway lead to cleavage of C4 and C2 resulting in formation of C3convertase.

(3) The alternative pathway is initiated by spontaneous hydrolysis of C3, leading to formation of C3a and C3b. If there is no pathogen C3a and C3b will rejoin . In the presence of pathogenic surfaces C3b will bind leading to the formation of C3 convertase of the alternative pathway.

All three pathways lead to activation of C3 in C3a and C3b. C3b contributes to the formation of C5 convertase, which initiates formation of the membrane attack complex.

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Nederlandse Samenvatting

[15]Wat is, was er reeds lang, en wat zijn zal, is reeds lang geweest; en God zoekt weer op, wat voorbijgegaan is.
prediker 3:15

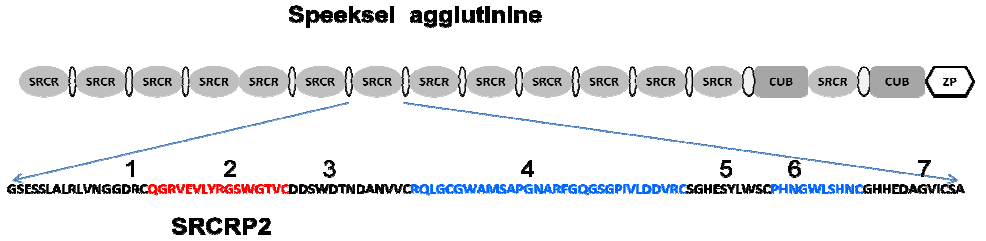
Inleiding

In de mond zijn honderden soorten bacteriën aanwezig in enorme aantallen; 1 ml speeksel bevat ongeveer 100 miljoen bacteriën. Ondanks deze enorme aantallen bacteriën hebben wij weinig last van infecties en met goede mondhygiëne kunnen wij ons leven lang doen met onze tanden. Anders wordt de situatie bij mensen die te weinig speeksel produceren, bijvoorbeeld na bestraling, bij medicijngebruik of bij het syndroom van Sjögren. Deze mensen hebben, naast problemen met spreken, kauwen en slikken, ook moeite met het gezond houden van de mond. De tanden zijn gevoeliger voor slijtage en cariës, en tong, verhemelte en wangen zijn gevoeliger voor infectie.
Om de mond gezond te houden bevat speeksel een hele set aan eiwitten die betrokken zijn bij de afweer. Deze eiwitten doden bacteriën of remmen de bacteriegroei, zoals lysozym, lactoferrine, histatinen en cystatinen. Daarnaast zijn er eiwitten die aan bacteriën binden en daarmee bacteriële hechting aan het tandoppervlak verhinderen, zoals agglutinine, S-IgA en MUC7. In dit proefschrift is de antimicrobiële werking van twee speekseleiwitten bestudeerd, speeksel agglutinine (SAG) en statherine.

Binding van SAG aan micro-organismen

Cariës is wereldwijd één van de meest voorkomende infectieziekten. Cariës wordt veroorzaakt door bacteriële omzetting van suikers in zuren. Door deze omzetting daalt de pH in de mond wat de groei van de meeste bacteriën remt. Frequente consumptie van suiker genereert een groot aantal langdurige zuurmomenten in de tandplak, waardoor bacteriën die ook nog bij lage pH doorgaan met zuurvorming uit suiker een selectief voordeel hebben en toenemen in aantal. Dit zijn de zogenaamde cariogene bacteriën die ervoor zorgen dat de zuurmomenten intenser worden en er cariës kan ontstaan. Eén van deze cariogene bacteriën is *Streptococcus mutans*. In het verleden is onderzocht welke speekseleiwitten kunnen binden aan *S. mutans* en daarmee mogelijk de kolonisatie beïnvloeden. SAG werd geïdentificeerd als één van de belangrijkste *S. mutans* bindende eiwitten in speeksel. SAG is een eiwit van ongeveer 340.000 dalton en bestaat voor een kwart van de massa uit koolhydraatketens. SAG komt niet alleen in speeksel voor, maar ook in tranen en longvloeistof, waar het bekend staat als gp340. SAG en gp340 worden beide afgelezen van het *DMBT1* gen op chromosoom 10. DMBT1 staat voor Deleted in Malignant Brain Tumours 1, omdat dit gen inactief is in bepaalde vormen van hersentumor. SAG behoort tot de Scavenger Receptor Cysteine Rich (SRCR) superfamilie van eiwitten. SRCR eiwitten komen in alle meercellige

dieren voor. Het zijn eiwitten met veel verschillende functies, die gemeenschappelijk hebben dat ze allemaal SRCR eiwitdomeinen bezitten. Eiwitdomeinen zijn geconserveerde stukjes eiwit die in verschillende eiwitten en organismen voorkomen. Op dit SRCR domein van 109 aminozuren ligt het bacterie-bindende gedeelte van SAG, een stukje van 16 aminozuren (Figuur 1). Wij vonden dat er een sterk verband is tussen de binding van een bacterie aan dit kleine gedeelte van dit eiwit (peptide) van 16 aminozuren en de binding aan het hele eiwit, wat erop wijst dat dit onderdeel een belangrijke bindingsplaats is voor bacteriën (Hoofdstuk 2). Door varianten van dit onderdeel te synthetiseren, waar telkens één aminozuur is vervangen door het aminozuur alanine, kon worden onderzocht hoe belangrijk elk aminozuur is voor de binding aan een bacterie. Door deze verschillende 16-aminozuur-lange peptiden te testen op een groot aantal bacteriën konden wij aantonen dat voor de binding van verschillende micro-organismen verschillende aminozuren essentieel zijn. Vier aminozuren waren echter altijd belangrijk voor de binding aan bacteriën. Drie daarvan zijn goed geconserveerd binnen andere SRCR eiwitten, namelijk tweemaal valine en eenmaal tryptofaan, wat erop wijst dat dit gedeelte ook in andere SRCR eiwitten een belangrijke functie heeft.



Figuur 1. Speeksel agglutinine is een eiwit dat bestaat uit SRCR domeinen, CUB domeinen en een zona pellucida (ZP) domein. Omdat het bacteriebindende gedeelte op de SRCR domeinen ligt zijn van het SRCR domein 7 fragmenten synthetisch gemaakt en getest op bacteriebinding. Alleen het tweede fragment (SRCRP2) is in staat bacteriën te binden.

Naast het binden van bacteriën is SAG ook in staat te binden aan de schimmel *Candida albicans* (Hoofdstuk 3). *Candida albicans* komt bij ca. 50 % van de bevolking voor in de mond, maar doet bij de meeste mensen weinig kwaad. Wanneer er echter problemen zijn met de afweer in de mond, zoals bij een tekort aan speeksel of bij gebruik van afweeronderdrukkende medicijnen, kan deze schimmel uitgroeien en infecties veroorzaken, in de volksmond bekend onder de naam spruw. Wij vonden dat SAG bindt aan *C. albicans*, maar dat er

ten opzichte van SAG binding aan bacteriën enkele essentiële verschillen zijn. In tegenstelling tot binding aan bacteriën was hechting aan *C. albicans* onafhankelijk van calcium-ionen en had chemische reductie van SAG geen invloed op de binding.

Remming van hyfenvorming van *Candida albicans*

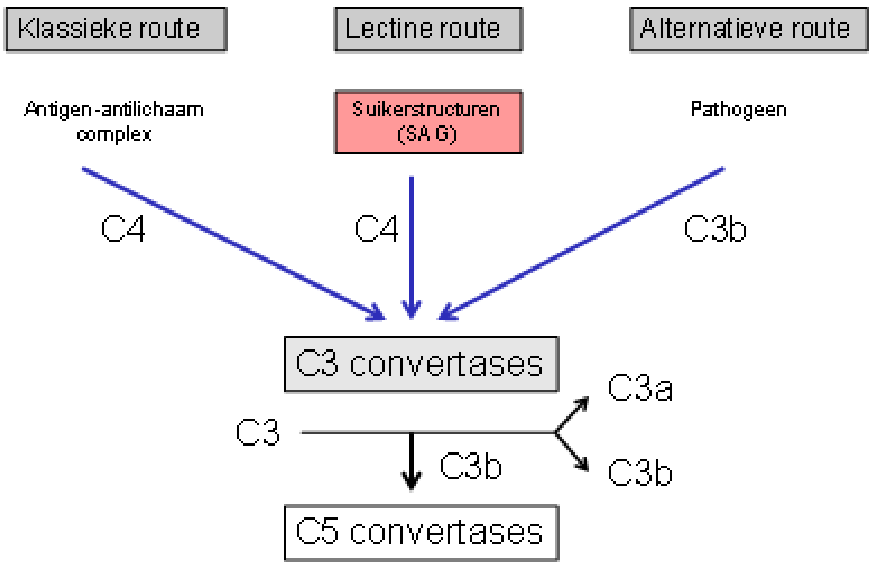
C. albicans komt in twee verschillende vormen voor; als ronde gistcellen en als draadvormige hyfen. De gistvorm hecht minder sterk aan diverse oppervlakken en kan zich makkelijk verspreiden. De hyfenvorm van *C. albicans* daarentegen hecht sterk aan diverse oppervlakken en kan in weefsel binnendringen en is daarom over het algemeen veel infectieuzer dan de gistvorm. In een gezonde mond komt *C. albicans* vrijwel alleen in de gistvorm voor. Daarom onderzochten wij of er in speekseleiwitten zijn die ervoor zorgen dat *C. albicans* als gistvorm groeit. Wij vonden dat het eiwit statherine ervoor zorgt dat *C. albicans* als gistvorm groeit onder omstandigheden die normaal hyfengroei veroorzaken. Statherine is een klein eiwit van 43 aminozuren. De eerste vijf aminozuren van het eiwit hebben een sterk negatieve lading. Hierdoor kan het eiwit goed calcium binden en speelt het een rol bij het herstellen (remineraliseren) van het tandglazuur na beschadiging door bijvoorbeeld zuur snoepgoed. Het eiwit is ook een belangrijke bindingsplaats voor bacteriën uit tandplaque. Door het eiwit systematisch in te korten vonden wij dat weglaten van de eerste 5 aminozuren de gistgroei versterkte. Door weglaten van dit fragment wordt het eiwit minder negatief geladen waardoor het sterker bindt aan het negatief geladen oppervlak van *C. albicans*. Verder inkorten toonde aan dat het statherine fragment waarin de eerste 10 aminozuren ontbreken de sterkste gistgroei gaf. De meeste fragmenten die een goede gistgroei veroorzaakten, waren ook in staat om hyfen los te weken van de kweekplaat wat zou kunnen wijzen op een gemeenschappelijke oorzaak voor beide verschijnselen. Er waren echter ook peptiden die hyfengroei remden, maar de hyfen niet losweekten van de kweekbodem, en peptiden die alleen de hyfen losweekten van de kweekbodem zonder gistgroei te veroorzaken. Dit wijst erop dat het stimuleren van gistgroei en het losweken van de kweekbodem twee verschillende oorzaken heeft.

Complement activering door SAG

Wanneer wij de concentratie van SAG in speeksel vergelijken met andere bacteriebindende eiwitten, zoals MUC7 en S-IgA, is die concentratie veel lager. Dit deed ons vermoeden dat remming van aanhechting niet de belangrijkste

functie is van SAG, maar dat SAG mogelijk een signaalfunctie heeft en bijvoorbeeld bepaalde afweerreacties activeert. Voorgaand onderzoek heeft aangetoond dat SAG in staat is het complementsysteem te activeren. Het complementsysteem is een systeem van serum eiwitten dat een belangrijke rol speelt bij de afweer tegen micro-organismen. Door binding aan micro-organismen worden enzymen geactiveerd, die weer een volgend enzym activeren. Uiteindelijk resulteert dit in fagocytose door immuun-cellen, aantrekken van immuun-cellen of directe doding van de micro-organismen. Er zijn drie manieren waarop het complement systeem geactiveerd kan worden.

(1) De klassieke route: Antigen-antilichaam complexen hechten aan C1q, het eerste eiwit wat bij de klassieke route een rol speelt.
(2) De lectine route: Mannose bindend lectine (MBL) of ficoline hecht aan suikerstructuren op het celoppervlak van een micro-organisme, wat een enzym activeert.
(3) De alternatieve route: Een derde mogelijkheid is dat het complement systeem zichzelf activeert door splitsing van het C3 eiwit in C3a en C3b. Wanneer er geen micro-organisme in de buurt is associeert C3 weer, maar wanneer er wel een micro-organisme aanwezig is bindt C3b zich covalent aan het micro-organisme wat ervoor zorgt dat de complementreactie wordt voortgezet op het oppervlak van het micro-organisme.



Figuur 2. Schematisch overzicht van de activering van het complement systeem. De klassieke route en lectine route leiden beide tot activering van C4. Activering van C4 leidt tot vorming van C3 convertase. De alternatieve route wordt geactiveerd door spontane hydrolyse van C3 die na binding met factor-B een C3 convertase vormt.

Voorgaand onderzoek toonde aan dat SAG het complement systeem activeert door binding aan C1q en dus waarschijnlijk de klassieke route van het complement systeem activeert. Door het meten van C4 depositie konden wij bevestigen dat SAG het complement systeem activeert. C4 is een eiwit wat een rol speelt bij de activering van het complementsysteem volgens de klassieke en lectine route, maar niet volgens de alternatieve route. Antilichamen tegen C1q, die de klassieke activeringsroute remmen, hadden weinig effect op de complement activering door SAG. Antilichamen tegen MBL waren echter wel in staat activering door SAG te remmen. Circa 20 % van de bevolking maakt geen of zeer geringe hoeveelheden MBL. Het complement systeem in serum van deze mensen werd nauwelijks geactiveerd door SAG. Deze gegevens wijzen erop dat SAG het complementsysteem via de MBL route activeert en niet via de klassieke route.
Om te achterhalen hoe SAG de MBL route activeert hebben we onderzocht of MBL suikerstructuren op SAG herkent. Hiervoor werd SAG met perjodaat bewerkt, wat suikerstructuren oxideert, en getest op complementactivering. Na

een perjodaat bewerking was SAG niet meer in staat het complementsysteem te activeren, wat erop wijst dat MBL suikerstructuren herkent op SAG. Het verdwijnen van de complement activering liep parallel met het verdwijnen van fucoseresiduen, wat erop wijst dat MBL waarschijnlijk aan fucoseresiduen op SAG bindt.

Conclusies

In dit onderzoek worden twee nieuwe beschermingsmechanismen in speeksel beschreven. Het eerste mechanisme is dat statherine de hyfengroei van *C. albicans* remt. Het tweede mechanisme is dat SAG in staat is het complementsysteem te activeren. Dit laatste mechanisme is een aanwijzing dat speeksel en bloed kunnen samenwerken bij de afweer tegen micro-organismen die via de mondholte ons lichaam binnendringen.

Acknowledgements / Dankwoord

[16]Toen ik mijn hart erop zette om wijsheid te leren kennen en om de bezigheid te aanschouwen, die op aarde geschiedt terwijl men noch bij dag noch bij nacht met zijn ogen de slaap te zien krijgt [17] zo zag ik, dat de mens niets kan ontdekken van het werk Gods, dat onder de zon geschiedt; want hoezeer de mens zich ook aftobt met zoeken, hij kan het niet ontdekken, en wanneer soms een wijze mocht zeggen, dat hij het weet, hij kan het niet ontdekken. Prediker 8: 16, 17

Acknowledgements / Dankwoord

Nu dat u dit boekje helemaal hebt "doorgelezen" en een goed beeld heeft van de wetenschappelijke inhoud ervan en zonder moeite met mij in discussie kan gaan over resultaten en conclusies en de validiteit van deze, wilt u natuurlijk ook weten met wie en hoe ik dit, zeer leerzame traject heb doorlopen.
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Bedankt voor uw aandacht en God zegene u

Thank you for your attention and God bless you

[28]Kom tot Mij, allen, die vermoeid en belast zijn, en Ik zal u rust geven; [29]neem mijn juk op je en leer van Mij, want Ik ben zachtmoedig en nederig van hart, en je zult rust vinden voor je ziel; [30]want mijn juk is zacht en mijn last is licht. Mat. 11:28-30