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Peptide Inhibitors of *Streptococcus mutans* in the Control of Dental Caries

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Peptides have been investigated as potential inhibitors of *Streptococcus mutans*, the main cause of dental caries, and have demonstrated considerable promise. In a human trial, topical application to tooth surfaces of a synthetic peptide inhibitor (p1025) of *S. mutans* adhesion prevented recolonisation with the oral pathogen following treatment with chlorhexidine gluconate (a broad spectrum antiseptic compound). An important feature of this treatment is that the duration of protection extends well beyond the period in which p1025 is applied. The specific targeting of *S. mutans* which allows other members of the oral flora associated with health to recolonise the oral cavity and competitively exclude *S. mutans* may explain the extended protection. Further i*n vitro* studies have identified several other peptides which may have potential as inhibitors of *S. mutans*. Of particular interest are studies that demonstrate that competence stimulating peptides of *S. mutans* act as inhibitors of *S. mutans* growth and that peptides derived from the competence stimulating peptides can be used as a means of specifically targeting broad spectrum antimicrobial peptides.

KEY WORDS: *Streptococcus mutans*; dental caries; bacterial adhesion; antimicrobials; streptococcal antigen I/II; adhesion blocking peptide; competence stimulating peptides; targeting peptides.

INTRODUCTION

Streptococcus mutans is the main aetiological agent of dental caries (Loesche et al., 1975; Loesche and Straffon, 1979). Colonisation of the tooth surface by this microorganism may be associated with production of lactic acid by fermentation of dietary carbohydrate. The reduction in local pH caused by lactic acid results in demineralisation of enamel. Thus strategies aimed at preventing infection of the oral cavity with *S. mutans* have been investigated as a means of preventing dental caries. Although *S. mutans* is susceptible to broad spectrum antimicrobial agents such as chlorhexidine gluconate (Emilson, 1994), recolonisation with *S. mutans* generally occurs when treatment is stopped. More effective protection against dental caries may be achieved by selectively removing *S. mutans* from the oral flora and exploiting the colonisation-inhibiting effect of competition from oral commensal flora for a limited ecological niche (Ma et al., 1989; Ma et al., 1990). The studies discussed in this review demonstrate the potential of peptide-based inhibitors of bacterial adhesion or cell growth for selective targeting of *S. mutans*.

MOLECULAR BASIS OF *S. MUTANS* ADHESION

A critical early step in microbial infection is attachment or adhesion to host tissue This process involves stereospecific interaction between molecules

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on the surface of the microorganism, termed adhesins, and receptors on host tissue. Adhesion of *S. mutans* to the tooth surface is mediated by a cell surface protein variously termed streptococcal antigen I/II (SA I/II) (Russell and Lehner, 1978), Pac (Okahashi et al., 1989a), antigen b (Russell, 1979) and SpaP (Lee et al., 1988) which binds to salivary glycoproteins adsorbed to the tooth surface. The salivary glycoprotein that forms the major receptor for *S. mutans* has been identified as gp-340, the lung scavenger receptor cysteine-rich protein (Prakobphol et al., 2000).

The importance of SA I/II in colonisation and caries prevention was demonstrated by *in vivo* studies in which monoclonal antibodies raised against SA I/II were applied directly to the teeth of rhesus monkeys which were fed on a human-type diet (Lehner et al., 1985). Monkeys treated with monoclonal antibody had significantly lower levels of colonisation with *S. mutans* and were protected against dental caries. In humans, topical application of the monoclonal antibodies also prevented colonisation (Ma et al., 1989; Ma et al., 1990). *In vitro* studies of spontaneously derived strains or isogenic mutants of *S. mutans* that are deficient in SA I/II demonstrated reduced binding to saliva-coated hydroxyapatite (McBride et al., 1984; Lee et al., 1989; Koga et al., 1990) confirming the role of the cell surface adhesin.

Sequence analyses of SA I/II and homologues from other species of oral streptococci (Okahashi et al., 1989b; Kelly et al., 1989; Ogier et al., 1990; Demuth et al., 1990; LaPolla et al., 1991; Tokuda et al., 1991) show a high degree of conservation. The main features of SA I/II derived from *S. mutans* strain NG5 (Kelly et al., 1989) are shown in Fig. 1. The N-terminal region includes 4 tandem repeats (3 well-conserved and one less conserved) of a sequence of 82 residues in which alanine is particularly abundant while the C-terminal region includes a series of 3 tandem repeats of a 39 residue proline-rich sequence. Further towards the C-terminal are a predicted bacterial cell wall-spanning sequence and a recognition motif (LPNTG) common to Gram positive bacteria for cleavage and covalent attachment of the C-terminus to the bacterial cell wall. Structure prediction and circular dichrosim spectroscopy (LaPolla et al., 1991; Demuth and Irvine, 2002) suggest that the alanine-rich sequences adopt an α-helical coiled coil conformation. The crystal structure of the fragment of SA I/II that is flanked by the alanine-rich and proline-rich repeats has been determined at a resolution of 2.4 Å (Troffer-Charlier et al., 2002) and comprises a lectin-like fold with an ion-binding site and a putative carbohydrate binding site.

ADHESION EPITOPE MAPPING OF SA I/II

To identify binding sites for salivary receptor within SA I/II, overlapping recombinant polypeptide fragments that span the complete sequence were expressed and assayed for binding activity (Munro et al., 1993). In an indirect assay in which inhibition of binding of intact *S. mutans* cells to saliva-coated hydroxyapatite was determined, only fragment 3 (spanning residues 821–1213) showed activity. Similar inhibitory activity was also demonstrated with a smaller fragment spanning residues 984–1161 of SA I/II (Kelly et al., 1995). Subsequently, in a study using surface plasmon resonance to measure direct binding to the salivary receptor, both the C-terminal fragment and fragment 2 (Fig. 1) bound with K_D values of approximately 10^{-8} M and 10^{-7} M, respectively (Kelly et al., 1999).

Adhesion epitopes within the C-terminal region were mapped further by preparing a panel of

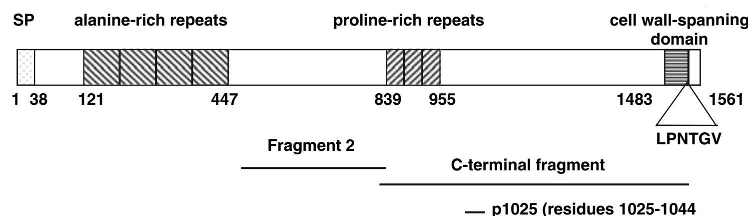

Fig. 1. Structure of SAI/II. The main features of the deduced amino acid sequence of SAI/II (Kelly et al., 1989) are shown together with the positions of recombinant polypeptide fragments and the peptide (p1025) spanning residues 1025–1044. Residues 1–38 form a predicted leader sequence (SP); residues 121–447 comprise 4 tandem repeats of an alanine-rich sequence; residues 839–955 are 3 tandem repeats of a proline-rich sequence while residues C-terminal to 1483 represent a predicted bacterial cell wall spanning region and include the sequence LPNTGV (1528–1533) a consensus signal for post-translational C-terminal modification resulting in cleavage and covalent attachment to the cell wall (Lee et al., 2002)

synthetic peptides (20-mers overlapping by 10 residues) spanning amino acid residues 803–1185 of SA I/II and measuring inhibition of binding of SA I/II to salivary receptor by surface plasmon resonance. Only the peptide spanning residues 1025–1044 (p1025: QLKTADLPAGRDETTSFVLV) was inhibitory in this system. Alanine-scanning in which each residue within p1025 was substituted in turn with alanine was performed to identify residues essential for binding activity. Substitution of residues Q1025, E1037 or any of the residues from 1041 to 1044 (FVLV) abolished or considerably reduced the inhibitory activity. The residues implicated in binding by this procedure were also mutagenised to alanine within the recombinant C-terminal polypeptide spanning residues 821–1538 of SA I/II (Fig. 1). Binding activity of the mutagenised polypeptides was determined by surface plasmon resonance which confirmed that Q1025 and E1037 contributed to SA I/II binding to salivary receptor but no effect on binding was evident when residues 1041–1044 were mutagenised. Nuclear magnetic resonance spectroscopic analysis of p1025 indicated that the peptide had no preferred solution conformation. It was suggested therefore that sequestration of the hydrophobic side chains of residues 1041–1044 may make an entropic contribution to the interaction between p1025 and salivary receptor. Taken together, these data provide evidence that the sequence of p1025 forms at least part of a major adhesion binding site of SA I/II.

HUMAN TRIAL OF P1025

In view of the finding that p1025 represents an adhesion epitope and inhibits *S. mutans* adhesion *in vitro*, a double blind trial was performed to determine whether p1025 was effective in preventing infection with *S. mutans* when applied topically to tooth surfaces in humans (Kelly et al., 1999). Following a procedure used previously for *in vivo* testing of monoclonal antibodies against SA I/II (Ma et al., 1989), volunteers were screened and those who carried *S. mutans* in the oral cavity were admitted to the trial. Subjects were treated with 0.2% chlorhexidine gluconate mouthwash and 1% chlorhexidine gel for 9 days, in order to reduce *S. mutans* to non-detectable levels. p1025, in the form of an acetylated peptide amide (10 mg/mL dissolved in 1.5%w/v $NaHCO_3$ in saline), or vehicle alone solutions were applied to the teeth of all subjects (5 µl/tooth surface, total volume of approximately 0.38 mL/subject). Six applications were performed over a period of 3 weeks. In addition, subjects were asked to use a mouthwash containing 1 mg/mL peptide or the NaHCO/saline solution daily for the first 2 weeks. Four subjects were given p1025, four were treated with $NaHCO_3$/saline only, and three were treated with a non-inhibitory peptide spanning residues 1125–1144 (p1125) of SA I/II. Recolonisation with *S. mutans* was determined by sampling of plaque and saliva at intervals as shown in Fig. 2.

In the group receiving p1025, no subjects had recolonised with *S. mutans* by 3 months after treatment and only one had recolonised by 4 months (Fig. 2a, b). In contrast, recolonisation was evident in subjects from both control groups (those receiving either p1125 or $NaHCO_3$/saline) by 2 months after treatment with chlorhexidine and all subjects in these groups had recolonised by 4 months after treatment. To investigate the specificity of treatment with p1025, the levels were investigated of another Gram-positive bacterium, *Actinomyces naeslundii*, that is also found commonly in the oral cavity. *A. naeslundii* was not detectable following treatment with chlorhexidine, however, recolonisation occurred in all subjects in the control group receiving p1125 and the experimental group treated with p1025 within 21 days of chlorhexidine treatment (Fig. 2c).

Mass spectrometric analysis of samples of saliva and washes of the tooth surface following application of p1025, indicated that the peptide was detectable in the oral cavity for only 4–6 h after application. Thus the relatively long term protection cannot be ascribed to persistence of p1025 in the oral cavity. A similar mechanism to that proposed for monoclonal antibody-mediated protection (Ma et al., 1989) may operate, namely that application with p1025 prevents initial colonisation with *S. mutans* and that recolonisation of the oral cavity with a flora associated with oral health provides long term protection by competitive exclusion of *S. mutans*.

IN VITRO STUDIES OF A GP340-DERIVED PEPTIDE INHIBITOR OF *S. MUTANS* ADHESION

The lung scavenger receptor cysteine-rich protein, gp340, was identified as the salivary agglutinin receptor for *S. mutans* (Prakobphol et al., 2000). The deduced amino acid sequence (Fig. 3) indicates that the N-terminal region of gp340 spanning residues 102–1740 (from a predicted sequence of 2431

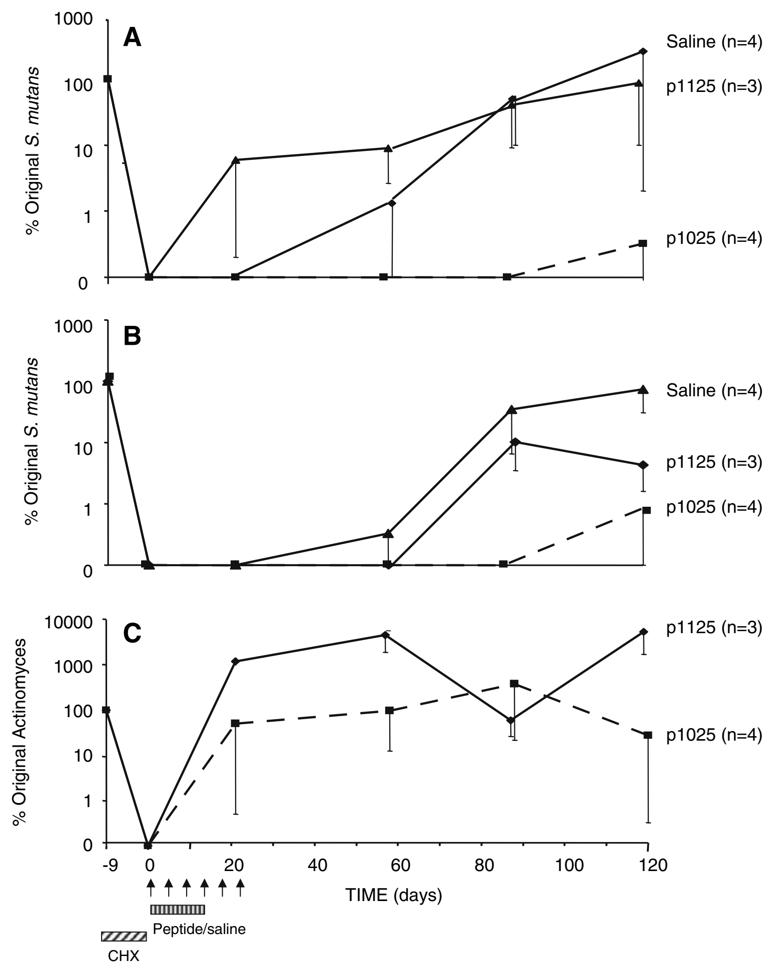

Fig. 2. Recolonization of *S. mutans* in the oral cavity in (A) dental plaque, (B) in saliva and (C) Actinomyces in dental plaque. Mean levels of bacteria (-sem) are plotted as percentages of the pre-experimental levels which are taken as 100%. Treatment with chlorhexidine gluconate (CHX) for 9 days (▨) is followed by six applications of peptide or control solutions (↑) and daily mouthwash (▥) (14 days). The three study groups each received saline/NaHCO3 (▲), p1125 (♦) or p1025 (■). Each group comprised four subjects except for the group receiving p1125 which had three subjects. (Reproduced with permission from Nature Biotechnology; Kelly et al., 1999)

residues) comprises 13 conserved tandem repeats of a 109 residue scavenger receptor cysteine-rich domain (SRCR) sequence with repeats being separated by short conserved SRCR-interspersed domains (SID) (Mollenhauer et al., 1997). Following digestion of gp340 with endoproteinase Lys-C, binding activity for *S. mutans* was associated with the large N-terminal fragment (that includes the SRCR repeats) predicted to be formed by cleavage at Lys 1812 (Bikker et al., 2002). Analyses of synthetic peptides that spanned consensus SRCR and SID sequences identified a single peptide, SRCRP2 (QGRVEV-LYRGSWGTVC; residues 18–33 of SRCR consensus repeat) with binding activity for *S. mutans*. The SRCRP2 peptide inhibited binding of *S. mutans* to gp340 immobilised on microtitre plates (approximately 70% inhibition at 200 μg/mL) and induced agglutination of the bacterium in suspension.

Binding activity of *S. mutans* cannot be ascribed solely to interaction with SRCRP2-like sequences since other studies have described carbohydrate-dependent binding associated with the adhesin SA I/II (Levine et al., 1978; Demuth et al., 1990b) and structural determination of a fragment of SA I/II has identified a putative lectin-binding conformation (Troffer-Charlier et al., 2002). Furthermore, the reported broad spectrum binding activity of the peptide (Bikker et al., 2002) does not provide a mechanism for the specificity of *S. mutans* adhesion. The lack of selectivity of the SRCR2 peptide may hinder its

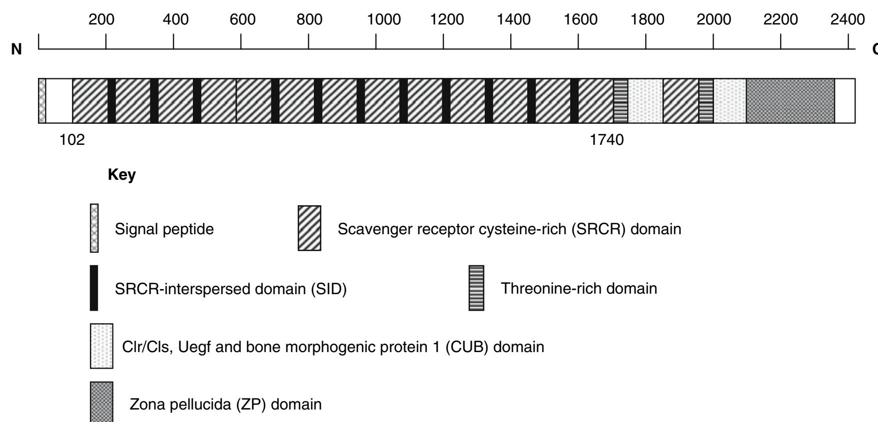

Fig. 3. Domain structure of salivary agglutinin/gp340. Amino acid residues are numbered. Note the presence of 13 tandem repeats of the SRCR domain separated by short sequences (SIDs) except between SRCR domains 4 and 5 which are contiguous. The SRCRP2 sequence is represented in each SRCR domain

potential use to block adhesion of *S. mutans* although (as discussed below) it may be possible to target the peptide by addition of competence stimulating peptide-derived sequences.

COMPETENCE STIMULATING PEPTIDES

Bacterial two-component signal transduction systems have attracted interest as potential targets for antimicrobial therapy (Stephenson and Hoch, 2002). Two component systems comprise a sensor histidine protein kinase that is activated specifically by an environmental signal and that in turn activates a specific transcription factor (response regulator) resulting in repression or activation of genes under its control. Induction of competence in *S. mutans* involves a two-component system (Li et al., 2001) and requires products of the three genes forming the *comCDE* locus (in addition to other gene products). The external ligand, the competence stimulating peptide (CSP) encoded by *comC*, is synthesised as a precursor that undergoes proteolytic processing to form the 21 residue CSP (SGSLSTFFRLFNRSF TQALGK) which is exported. CSP binds to the membrane-bound histidine kinase sensor (*encoded by comD*) resulting in autophosphorylation of the histidine kinase. The phosphate group is in turn transferred to the intracellular response regulator (encoded by *comE*) which leads to expression of further competence-related genes.

Reasoning from studies that demonstrated an enhanced stress response to CSP, Qi et al. (2005) tested the effect on *S. mutans* of CSP at concentrations exceeding that required for the induction of competence. Two strains of *S. mutans* were tested and growth of both strains in both planktonic and biofilm cultures was inhibited by addition of CSP while no inhibition was evident for three other species of closely related oral streptococci. From microscopic examination, it was proposed that CSP acted to inhibit cell division leading to cell death. Mutagenised strains of *S. mutans* lacking *comD* or *comE* confirmed that the respective gene products were essential for growth inhibition. The exquisite species-specificity of the CSP is a particular advantage of this approach to inhibit *S. mutans*. Furthermore, there appears to be limited genetic variability in *comC* within *S. mutans*. In a study of 36 geographically diverse strains, 7 *comC* alleles were identified encoding 3 conserved but distinct CSPs (Allan et al., 2007), however, all CSP variant strains responded (i.e. showed increased transformation frequency) in an identical manner to each CSP. Thus it is likely that a wide range of *S. mutans* strains can be targeted by a single CSP, although examination of more strains will be necessary to rule out strain differences in susceptibility. Use of CSPs to selectively inhibit bacteria may be applied more generally since an earlier study (Oggioni et al., 2004) demonstrated efficacy of a CSP from *Streptococcus pneumoniae* in a murine model of sepsis mediated by the bacterium.

An interesting development of this approach is to use the CSP as a targeting moiety and to couple it to a broad spectrum antimicrobial. A synthetic peptide, C16G2, comprising the 16 C-terminal residues of the *S. mutans* CSP linked by a short spacer (GGG) to a 16 residue sequence derived from the broad spectrum

antimicrobial peptide novispirin was active against *S. mutans* (Eckert et al., 2006). The peptide C16G2 (sequence: TFFRLFNRSFTQALGKGGGKNL-RIIRKGIHIIKKY) killed planktonic *S. mutans* with minimum inhibitory concentration (MIC) 3–4 fold lower than that of the antimicrobial peptide alone. In this assay, C16G2 was also active against two other species of oral streptococci although with MIC higher than that required for inhibition of *S. mutans* (approximately 19 and 23 μM for *Streptococcus sanguinis* and *Streptococcus gordonii*, respectively, compared with 3–5 μM for *S. mutans*). Presumably this is due to prolonged incubation with the antimicrobial peptide conjugate since the selectivity for *S. mutans* was greatly increased when bacteria were exposed to C16G2 for only a brief priod (1 min). In addition, the peptide showed good selectivity and activity against *S. mutans* in biofilms. In a further development, a shorter sequence of 8 residues (TFFRLFNR) derived from the CSP was identified as sufficient for targeting the antimicrobial peptide with obvious benefits for ease of production of peptide inhibitors.

The targeted peptide inhibits bacterial growth by a mechanism that is *comD*-independent and therefore is likely to require an alternative receptor (*Eckert* et al., 2006). The mechanism appears therefore to be distinct from that of CSP administered alone (Qi et al., 2005) and may not involve induction of the competence phenotype.

CONCLUDING REMARKS

The demonstration that topical application of p1025 prevented recolonisation with *S. mutans* in humans (following treatment with a broad spectrum antiseptic) indicates the potential for use of peptides to prevent infection in the oral cavity. Despite the flow of saliva and the likely presence of host-derived or microbial-derived proteases, p1025 persistence in the oral cavity was sufficient to prevent recolonisation. In this study and others discussed above, the selective inhibition of *S. mutans* following treatment with a broad spectrum antimicrobial demonstrated a clear advantage over the use of broad spectrum antimicrobials alone. Treatment with a specific inhibitor of *S. mutans* allows recolonisation of the oral cavity with a flora associated with health that may competitively exclude the oral pathogen. The protective effect therefore lasts considerably longer than the period of application of the targeted inhibitor.

Salivary agglutinin/gp340 interacts with various species of oral streptococci mediating both aggregation in fluid-phase and adhesion when surface-bound (Prakobphol et al., 2000; Loimaranta et al., 2005). The inhibitory effect of p1025 on adhesion has only been reported for *S. mutans* although the peptide inhibits gp340-mediated aggregation of a strain of *Lactococcus lactis* engineered to express, on the cell surface, the SA I/II homologue adhesin (termed SspA) of *Streptococcus gordonii* (Jakubovics et al., 2005). Similarly, the corresponding peptide from SspA, p1023 (spanning residues 1023–1042) inhibited gp340-mediated aggregation of a strain of *L. lactis* engineered to express SA I/II on the cell surface. In the region of SspA corresponding to the p1025 sequence, there are 6 substitutions compared with *S. mutans*. It is likely, therefore, that p1025 may also inhibit adhesion of species of oral streptococci other than *S. mutans*. However, long term prevention of colonisation is likely to depend not only on susceptibility to an adhesion-inhibitor but also on the ability to compete with other members of the oral flora.

Peptide-based anti-infective agents have the advantage of being relatively simple to engineer for improved activity and specificity. In the latter regard, the studies with CSP and CSP-derived peptides from *S. mutans* provide a potentially powerful platform for highly specific targeting of broad spectrum antimicrobial peptides again with the aim of inhibiting *S. mutans* but not commensal oral bacteria. Development of peptide-based treatments for prevention of dental caries could result in demand for very large quantities of peptide, possibly in the range of tonnes per year. Although the costs of large scale synthesis of peptides have been regarded as prohibitive, the development of peptide drugs such as the HIV-1 fusion inhibitor Fuzeon of which more than 3.5 tonnes are synthesised annually using new processes for large scale synthesis (Bray, 2003) has significantly lowered costs.

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