Multivariate Design and Evaluation of a Set of RGRPQ-derived Innate Immunity Peptides

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Oral commensal Streptococcus gordonii proteolytically cleave the salivary PRP-1 polypeptide into an RGRPQ innate peptide. The Arg and Gln termini are crucial for RGRPQ-mediated ammonia production and proliferation by S. gordonii SK12 and adhesion inhibition and desorption by Actinomyces naeslundii T14V, respectively. Here we have applied (i) a multivariate approach using RGRPQ-related peptides varied at amino acids 2, 3, and 4 simultaneously and (ii) size and N- and C-terminal modifications of RGRPQ to generate structure activity information. While the N-terminal arginine motif mediated ammonia production independent of peptide size, other responses required more or less full-length peptide motifs. The motifs for adhesion inhibition and desorption were the same. The adhesion and proliferation motifs required similarly a hydrophobic/low polarity amino acid 4 but differentially a hydrophilic or hydrophobic character of amino acids 2/3, respectively; polar peptides with small/hydrophilic and hydrophilic amino acids 2 and 3, respectively, had high adhesion inhibition/desorption activity, and lipophilic peptides with large/hydrophobic amino acids 2 and 3 had high proliferation activity. Accordingly, while RIWWQ had increased proliferation but abolished adhesion/desorption activity, peptides designed with hydrophilic amino acids 2 and 3 were predicted to behave in the opposite way. Moreover, a RGRPQ mimic for all three responses should mimic small hydrophilic, large nitrogen-containing, and hydrophobic/low polarity amino acids 2, 3, and 4, respectively. Peptides fulfilling these criteria were 1–1.6-fold improved in all three responses. Thus, both mimetics and peptides with differential proliferation and adhesion activities may be generated for evaluation in biofilm models.

Saliva confers infection resistance and wound healing to oral tissue surfaces (1, 2). The saliva innate defense resides in general properties, such as clearance and pH, and specific innate (poly)peptide functions. These innate peptide functions control adhesion and colonization of commensal and pathogenic microorganisms as well as tissue homeostasis (3, 4).

Acidic, basic, and glycosylated proline-rich proteins (PRPs), encoded by six genes on chromosome 12p13.2 (S), are abundant and polymorphic proteins in saliva (1, 6). Acidic PRPs mediate adhesion of commensal Streptococcus and Actinomyces species (7, 8), neutralize dietary tannins (polyphenols), and interact with calcium (1). These interactions occur through the C-terminal, proline-rich middle and phosphorylated N-terminal domains, respectively (1). PRPs are subject to endogenous and bacterial proteolysis, generating a wide range of peptide derivatives in saliva (6, 9, 10). Both allelic PRP variants and small size peptides derived thereof coincide with susceptibility or resistance to caries (11, 12). The allelic acidic PRP variant Db coincides with caries susceptibility and adhesion of Streptococcus mutans (12), implicated in caries. The other acidic PRP variants (e.g. PRP-1 and PRP-2) coincide with resistance to caries and adhesion of commensal streptococci and actinomyces (12). Moreover, commensal Streptococcus gordonii SK12 proteolytically cleave PRP-1 into a multipotent RGRPQ peptide that regulates plaque pH in situ, stimulates proliferation of the same organism, and inhibits adhesion of Actinomyces naeslundii T14V, which competes for acidic PRP binding sites (10, 13). The effect on plaque pH by RGRPQ resides in inhibition of bacterial acid production from sucrose and catabolism of arginine to ammonia. The RGRPQ peptide could, similar to the ERGMT and ARNQT peptide signals that affect intra- or extracellular receptors and gene expression in Bacillus subtilis (14), mimic natural peptide signaling substances in the streptococcal biofilm. In S. gordonii challis, genes encoding peptide pheromone receptors are present (15).

We have shown that the Arg and Gln termini of the RGRPQ peptide are crucially, though differentially, linked to the various innate responses by systematically replacing one amino acid after another with alanine and comparing the activities of designed and wild type peptides (13). Such alanine, proline, or serine scans represent simple and easy strategies to uncover structure-activity relationships. A more powerful approach is to use statistical molecular design (SMD), an experimental design approach (16, 17), in combination with quantitative structure activity-relationship (QSAR) analyses based on quantitative amino acid descriptors (18, 19). QSARs are modeled using multivariate data analysis techniques, such as partial least squares projection to latent structures (PLS) (20, 21). In SMD and QSAR, several amino acid positions are varied simultaneously. The SMD and QSAR approach reveals both amino acid properties and interactions between amino acids pertinent to activity and, consequently, forms a platform for the generation of peptide mimetics. This approach has, for example, been used to develop mimetics that inhibits pili assembly and urinary tract infections based on the interaction of pili chaperones with peptides from the cell surface pilus subunit of uropathogenic Escherichia coli (22, 23).

The aim of this study was to apply (i) SMD and PLS to establish QSARs for a set of RGRPQ-related peptides varied at amino acids 2–4 simultaneously and (ii) size and N- and C-terminal modifications of RGRPQ to generate structure activity information. The results were used to design new peptides with differential adhesion inhibition/desorption and proliferation activities. Moreover, this SMD approach extracted which properties in the various positions in the peptides that controlled the various biological
responses. The results therefore constitute a solid platform for extended studies toward novel peptidomimetics.

**EXPERIMENTAL PROCEDURES**

**Peptides**—Peptides were synthesized, purified using high performance liquid chromatography, and characterized by mass spectrometry (Thermo Electron GmbH, Ulm, Germany). Peptides were dissolved in sterile water, adjusted to pH 6.8–7.0, and frozen as aliquots at −20 °C prior to the experiments. All peptides were fully soluble at the concentrations used for the biological assays.

**SMD**—SMD (16, 17) is an approach for selection of subsets of compounds from large virtual compound libraries. It can, for instance, be used to select well balanced compound sets and thereby reducing the number of compounds required for establishing QSARs. An initial step in SMD and QSAR is to quantitatively describe the compounds, i.e. peptide sequences, with molecular descriptors. Here the z-scales for hydrophilicity ($z_1$), size ($z_2$), and electronic effects ($z_3$) were used to quantitatively describe the separate amino acids in the sequence (26). In addition, lipophilicity (SlogP) and total polar surface area (TPSA) (26) were calculated in MOE (27), for the entire peptide, resulting in a so-called descriptor matrix with 11 variables for each peptide sequence (Fig. 1).

In this work, a D-optimal design (28) was used to select a set of 14 diverse peptides for QSAR modeling. While amino acids 2–4 of the RGRPQ peptide were varied simultaneously using all 20 natural amino acids in the candidate set used for SMD, the functionally crucial Arg and Gln were kept constant. This resulted in a candidate set of 203, or 8,000, RXXXXQ peptides. The candidate set was reduced to 7,600 peptides by exclusion of the most distant or extreme five percent of the peptides, as described quantitatively using the z-scales for hydrophilicity ($z_1$), size ($z_2$) and polarity (or electronic effects) ($z_3$), and peptide properties using SlogP (lipophilicity) and TPSA. The effect of some of the designed peptides on proliferation of S. gordonii SK12 was therefore studied, providing a solid platform for extended peptidomimetics.

**QSAR Modeling**—The method of PLS was used to calculate the QSAR model for the 14 test peptides and three biological responses (SIMCA-p + 10.0) (31). The PLS method identifies the information in $X$ (amino acid properties) that relates to the variation in $Y$ (biological responses), i.e. the amino acid properties that result in a change in response. $R^2$ and $Q^2$ are used to diagnose the model. $R^2$ is a measure of how much of the variation in $Y$ that is explained by the model, and $Q^2$ is a measure of the cross-validated predictive ability of the model. $R^2$ and $Q^2$ values can also be generated for each separate response in the model. The QSAR model was used to predict the biological activity for the remaining 7,585 pentapeptides in the candidate set not selected by the D-optimal design. Six of these peptides were tested to verify the reliability of the QSAR-model, i.e. external validation of the model.

**Bacterial Strains**—S. gordonii SK12 and A. naeslundii T14V (8, 32) were grown overnight on Columbia-II-agar base plates (BD Biosciences), supplemented with 30 ml of a human erythrocyte suspension per liter, at 37 °C and 5% CO₂. The strains intended for adhesion inhibition tests were metabolically labeled with [¹⁵N]methionine (8).

**Growth-inducing Capacity (Proliferation)**—The ability of RGRPQ-related peptides to induce growth of S. gordonii strain SK12 (referred to as proliferation) was measured by culturing SK12 in a minimum growth medium supplemented with peptide. The minimum growth medium, generated by dilution eight times of a chemically defined medium (free amino acid pool; Ref. 33), was filter-sterilized (Millipore) and aliquoted (100 μl/well) into a 96-well microtiter plate. Prior to inoculation of strain SK12 to the minimum growth medium, SK12 was cultured twice and inoculated to 0.05 optical density.
and consecutively in chemically defined medium at 37 °C and 5% CO₂. The second culture was grown for 14 h and used as an inoculum (1%, 1/100) to the minimum growth medium supplemented with 150 μM peptide (peptide/free amino acid pool ratio was 1:20). The effect by RGRPQ on growth was dose-dependent between 25 and 250 μM peptide, and 150 μM RGRPQ increased the regeneration time from 4.35 to 1.85 h. The inoculated wells were incubated for 18 h at 37 °C and 5% CO₂. Bacterial cell numbers were established by recording the absorbance at 550 nm using a SpectraMAX 340 spectrometer (Molecular Devices, Sunnyvale, CA) before and during culturing. Absorbance values within the range of the assay were linearly proportional to the bacterial cell concentration, as established by correlating the absorbance values and bacterial cell numbers obtained from counting of cells on agar plates.

Adhesion Inhibition—Inhibition of adhesion of A. naeslundii T14V to PRP-1 was performed by preincubation of cells with peptides (final concentration of 10 mM) for 20 min at room temperature prior to adhesion tests. The adhesion test of 35S-labeled A. naeslundii T14V to PRP-1 coated onto hydroxyapatite beads was performed essentially as described (8 mg of beads, 5 × 10⁶ cells/ml and 1-h adhesion) (8).
Reversal of Aggregates of Bacteria and PRP-1-coated Beads (Desorption)—The ability of RGRPQ-related peptides to reverse aggregates between A. naeslundii T14V and PRP-1-coated latex beads (referred to as desorption) was measured by adding peptides to preformed aggregates. Aggregates of A. naeslundii T14V and PRP-1-coated latex beads were formed as described (10), except for the use of 6 \times 10^7 cells/ml of bacterial suspension. Bovine serum albumin-coated latex beads served as a control and did not induce aggregates. Untreated control strain T14V induced strong aggregation (i.e. clumping of aggregates in clear solution, score 0 below) of bacteria and PRP-1-latex beads. Aggregates were reversed by adding peptide at a total concentration of 5.06 mM, and the degree of desorption was related to the T14V control (without peptide and visually scored from 0 to 5 (no, weak, moderate, strong, very strong, and complete desorption, respectively) based on the following criteria: 0, clumping of aggregates in clear solution; 1, large aggregates in clear solution; 2, moderate aggregates in clear solution; 3, moderate aggregates in unclear solution; 4, small aggregates in unclear solution; and 5, no visible aggregates.

Ammonia Production—An overnight culture of S. gordonii SK12 was washed once in 10 ml sterile water, adjusted to 4.8 \times 10^7 cells/ml with water, and kept on ice for a minimum of 30 min before use (34). Bacterial cells (90 \mu l) were mixed with test peptide, and sterile water was added to give a final volume of 100 \mu l at a final peptide concentration of 0.5 mM. The mixture and control cell suspension were incubated at 37 °C aerobically, and pH was measured using a pH-electrode.

RESULTS

QSARs of RGRPQ-related Peptides for Three Biological Responses—The PLS method was used to generate a QSAR model from a set of 14 RGRPQ-related peptides varied at amino acids 2–4 simultaneously and their proliferation, adhesion inhibition, or desorption activities (Figs. 1 and 2). The 14 diverse, and for the quantitative properties representative, designed test peptides are shown in Fig. 3A. The PLS (QSAR) model (R^2 = 89%, Q^2 = 69%) showed the relationship of single (linear terms) or interacting (interaction terms) amino acid properties of the peptides with the three biological responses (Fig. 2). Molecular structures and a schematic representation of the structure activity relationships are given in Figs. 4 and 5.

The R^2 and Q^2 for the separate responses showed similar values, leading to the conclusion that they were all explained well by the model: proliferation (R^2 = 92%, Q^2 = 71%), adhesion inhibition (R^2 = 86%, Q^2 = 69%) and desorption (R^2 = 87%, Q^2 = 60%).

Amino Acid and RGRPQ Characteristics Influencing Proliferation Activity—The proliferation response depended on similar properties as for adhesion inhibition/desorption for amino acid 4 but different properties for amino acids 2 and 3 (Fig. 2). The following properties of amino acids 2–4 or the overall peptide correlated with a high proliferation activity (Fig. 2A): (i) large (z2) and hydrophobic (z1) amino acids 2 and 3 as illustrated by the potent RWWCQ and RWWHQ peptides with hydrophobic tryptophan (Trp) or isoleucine (Ile) groups at these positions, (ii) a hydrophobic and low polarity of amino acid 4 (z3–4) (e.g. cysteine), and (iii) a high overall peptide lipophilicity, SlogP, as illustrated by the low potency of the RKKQ peptide with a charged residue in all three positions.

Some amino acid or peptide properties interacted (i.e. interaction terms) with a large size of amino acid 3 (z2-3) to generate a high proliferation activity (Fig. 2A): (i) a high peptide lipophilicity (SlogP^P*z2-3), (ii) high hydrophobicity for amino acid 3 (z1-3*z2-3), and (iii) low polarity of amino acid 4 (z2-3*z3-4). The strength of these interactions and the highly influential and linear dependence on hydrophobic properties of amino acid 3 (z1-3) emphasizes the primary importance of a large and hydrophobic amino acid 3 that largely guides the properties of amino acids 2 and 4 (e.g. if increasing the size of amino acid 3 above a threshold level, the size of amino acid 4 should be diminished to retain high activity).

Some peptides mediated 1–1.6-fold increased proliferation (e.g. RWWCQ and RIWWQ) compared with RGRPQ, while others (e.g. Figs. 3A and 3B) show the predicted position of six peptides used for verification of the model on proliferation, adhesion inhibition, and desorption. Two of the peptides selected for high proliferation activity (RPWCQ and RWWHQ) and two selected for high adhesion inhibition/desorption activity (RGRQ and RGWAQ) displayed improved activity compared with RGRPQ and the corresponding responses.

![Figures](image-url)
RKKKQ or RDDDQ) displayed reduced but still some proliferation activity (Fig. 1).

**Virtually Identical RGRPQ Properties Determine Adhesion Inhibition and Desorption Activity**—The PLS (QSAR) model showed adhesion inhibition and desorption to depend on virtually identical amino acid properties (Fig. 2, B and C), as illustrated by RGHPQ and RGRPQ showing high adhesion inhibition and desorption activities, while other peptides were less potent for both responses. The following amino acid properties coincided accordingly with both a high adhesion inhibition and desorption activity (Fig. 2, B and C): (i) a hydrophobicity and low polarity of amino acid 4, (ii) a large size and hydrophilicity of amino acid 3, as illustrated by the potent RGRPQ and RGHPQ peptides with a bulky and nitrogen-containing arginine (Arg) or histidine (His), and (iii) hydrophilicity and small size of amino acid 2, as illustrated for the potent RGRPQ and RGHPQ peptides with a glycine at this position. Substitution of glycine with sterically demanding or charged residues as for RIWWQ and RDDDQ, respectively, abolished accordingly the activity.

Some amino acid or peptide properties interacted (i.e. interaction terms) with a small size of amino acid 2 (z2-2) to generate a high adhesion inhibition or desorption activity (Fig. 2, B and C): a hydrophilic amino acid 3 (z2-2*z1-3) and amino acid 2 (z1-2*z2-2) and high total polar surface area (TPSA*z2-2). Accordingly, the positive z1-2*z2-2 terms for both adhesion inhibition/desorption and proliferation, which show inversed correlations for the corresponding linear properties, emphasize that size and hydrophobic/hydrophilic character of amino acid 2 affects the two responses differently. Similarly, while a hydrophilic character of amino acid 3 (TPSA*z1-3) correlates with a high adhesion/desorption activity, high proliferation activity depends on a large and hydrophobic character of the same amino acid (SlogP*z2-3 and z1-3*z2-3). Thus, peptides with differential proliferation and adhesion inhibition activities can be designed.

**Peptides Designed from the QSAR Model Behaved as Expected**—To verify the QSAR model and delineated structure-activity relationships, we tested the activity of six peptides predicted to have either a high proliferation or adhesion inhibition activity (Fig. 3, A and B). The RPWCQ and RWWHQ peptides mediated as predicted high proliferation but not adhesion inhibition/desorption and contained a large hydrophobic proline (Pro) or tryptophan (Trp) in position 2, tryptophan in position 3, and low polarity cysteine or histidine (His) in position 4, respectively. The RGWAQ and RGRQ peptide, on the other hand, showed as predicted a high adhesion inhibition and desorption activity and, accordingly, contained a small glycine (Gly) in position 2, a large nitrogen containing tryptophan (Trp), or arginine (Arg) in position 3 and a low polarity alanine (Ala) or cysteine (Cys) at position 4 (see Figs. 4 and 5). Thus, peptides with differential proliferation and adhesion inhibition/desorption activities were found.

In addition, the RGRQ and RGWAQ verification peptides and RGHPQ test peptide were improved in all three responses as compared with RGRPQ (Fig. 3B and supplemental Table 1). The three peptides are characterized by (i) a small amino acid 2 (Gly), (ii) a large, hydrophobic and nitrogen-containing amino acid 3 (Trp), and (iii) a hydrophobic (Ala) or low polarity (Cys) amino acid 4 (Fig. 5).

**Effects of N- and C-terminal and Size Modifications of RGRPQ on Its Innate Properties**—We next used N- and C-terminal and size modifications of the RGRPQ peptide to further explore peptide characteristics essential to the RGRPQ innate properties (Table 1). Adhesion inhibition and desorption resided largely in the GRPQ sequence, since both PQ and RPQ lacked inhibitory activity and since the N-terminal Arg marginally increased the activity of the RGRPQ
peptide. Moreover, the unmodified RGRPQ peptide was more active than the C- or N-terminally modified peptides, although the C-terminal amidation of Gln, which removes its negative charge, reduced the activity more profoundly than the N-terminal acetylation.

Stimulation of proliferation resided more or less in the full-length RGRPQ sequence, since PQ lacked activity, and since high but somewhat increasing activity occurred in the order of RGRPQ > GRPQ > RPQ. The RPQ tripeptide had markedly high proliferation activity compared with its low adhesion activity. Both C- and N-terminal modifications of RGRPQ reduced the proliferation activity dramatically. Moreover, di- or tri-repetitive RGRPQ peptides (RGRPQ₂ and RGRPQ₃) were as potent stimulators as the RGRPQ peptide.

Ammonia production occurred rather independently of size. The RG and AGR peptides and the di- or tri-repetitive RGRPQ peptides (RGRPQ₂ and RGRPQ₃) showed activities rather similar to full-length RGRPQ. Moreover, ammonia production occurred rather independently of an N- or C-terminal location of the Arg residue. Finally, while the activity was completely blocked by acetylation of the N-terminal Arg residue, amidation of the C terminus of RGRPQ did not affect activity.

**DISCUSSION**

This study shows that multivariate design and evaluation of small sized RGRPQ-related peptides generates valuable information on amino acid properties essential for its activity and for the generation of peptide mimetics or mimetic building blocks. The RGRPQ peptide is challenging for mimetic development as it affects proliferation, adhesion, and local pH, which all are key components in biofilm formation and should be retained by a peptidomimetic. An RGRPQ mimetic platform for all three responses should mimic a small hydrophilic, a large nitrogen-containing (potentially involved in H-bonding), and a hydrophobic/low polarity amino acids 2, 3, and 4, respectively, as well as arginine (Arg) and glutamine (Gln) at positions 1 and 5 (Fig. 5). The RGHPQ, RGRCQ, and RGWAQ peptides, which fulfilled these criteria, showed slightly enhanced activities for proliferation and adhesion inhibition/desorption. Moreover, the specific mimetic platforms for inhibition/desorption and proliferation are similar for amino acids 4 and 5, while different for amino acids 2 and 3 (Fig. 5). The QSAR model from the small panel of designed peptides allowed accordingly a large volume of information, and to our delight, peptides predicted from the model showed the expected activity. An even larger set of peptides with systematic variations at amino acids 1–5 would, consequently, provide an even better guidance to the generation of RGRPQ mimetics to control microbial ecology and disease development. In this respect, it is noteworthy that the RGRPQ peptide supports oral colonization of *S. gordonii* SK12 in rats in vivo (13), potentially by inducing a more rapidly growing bacterial phenotype (14).

The present results suggest a blend of complex and simple peptide motifs for proliferation, adhesion inhibition, and ammonia production. The complex, though differential, peptide motifs for proliferation and adhesion inhibition/desorption involved more or less the full-length RGRPQ peptide. A high proliferation activity is mediated by RGRPQ, GRPQ, and RPQ (RGRPQ and GRPQ > RPQ) and peptides with
unmodified glutamine (Gln), large and hydrophobic properties of amino acids 2 and 3, and hydrophobic and low polarity properties of amino acid 4 (Fig. 5). The importance of a high overall peptide lipophilicity, largely involving amino acid 3, may relate to uptake of the peptide at the cellular membrane or to the character of intra or extra cellular receptors. Moreover, a high adhesion inhibition/desorption activity is mediated by GRPQ with an unmodified Gln terminus, a small amino acid at position 2 (Gly) and a hydrophobic/lowlow polarity (Pro or Cys) amino acid at position 4. Position 3, on the other hand, allowed a variety of substituents but should favorable be slightly hydrophilic (Fig. 5). The PQ and RPQ peptides lacked inhibitory activity and conformational features of glycine at position 2, potentially guiding a cis or trans configuration of proline (Pro), may accordingly contribute to the high GRPQ activity. The virtually identical requirements for adhesion inhibition and desorption are consistent with two related phenomena but surprising, since they are surface versus solution reactions, respectively. Bacterial desorption or release of multiple fimbriae-receptor interactions may, however, follow solution kinetics in general or reflect the aggregation assay used, as opposed to irreversible protein ligand interactions at surfaces, e.g. antibody-antigen surface reactions (24). Finally, ammonia production required only a simple arginine (Arg) motif independent of peptide size or N- and C-terminal localization. It is possible that arginine protrudes from the rest of the RGRPQ peptide, which largely accounts for proliferation and adhesion inhibition/desorption (i.e. GRPQ), and that the arginine deaminase (involved in the catabolism of arginine to ammonia by bacteria) recognizes the protruding amino acid 4 (Fig. 5).

Adhesion and proliferation of microorganisms and local conditions, such as pH, are key factors in biofilm formation (25). In oral biofilm formation, frequent intake of sugar induce acid production from bacteria and a pH decrease changing the biofilm ecology and its potential to cause caries. It is certainly interesting that the RGRPQ peptide is capable of affecting adhesion, proliferation, and pH and that the present findings show the possibility of designing peptides that affects these properties selectively. While RIWWQ increased proliferation but lacked adhesion inhibition/desorption activity, the QSAR model predicts peptides with even larger differences and with the opposite behaviors. While a hydrophobic amino acid 3 and peptide character promotes proliferation selectively, a hydrophilic amino acid 3 and peptide character promotes adhesion inhibition selectively (Fig. 5). There has been a long standing question of whether compounds affecting proliferation versus adhesion are more effective in controlling biofilm formation and infectious diseases in vivo. This study enlightens the possibility to design RGRPQ derivatives with differential adhesion and proliferation activities for evaluation in biofilm models.

In conclusion, this study has drug development implications and provides an example of statistical molecular design as a platform to extract important structure activity relationships from peptides with a multitude of biological properties.

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