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Screening and Characterization of Surface-Tethered Cationic Peptides for Antimicrobial Activity

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SUMMARY

There is an urgent need to coat the surfaces of medical devices, including implants, with antimicrobial agents to reduce the risk of infection. A peptide array technology was modified to permit the screening of short peptides for antimicrobial activity while tethered to a surface. Cellulose-amino-hydroxypropyl ether (CAPE) linker chemistry was used to synthesize, on a cellulose support, peptides that remained covalently bound during biological assays. Among 122 tested sequences, the best surface-tethered 9-, 12-, and 13-mer peptides were found to be highly antimicrobial against bacteria and fungi, as confirmed using alternative surface materials and coupling strategies as well as coupling through the C and N termini of the peptides. Structure-activity modeling of the structural features determining the activity of tethered peptides indicated that the extent and positioning of positive charges and hydrophobic residues were influential in determining activity.

INTRODUCTION

The rapid progress of biomedical technology and an aging population places increasing demands on medical implants to treat serious tissue disorders and replace organ function. In the field of orthopedic implant surgery alone, about 2 million fracture-fixation devices and 600,000 joint prostheses are implanted every year in the United States (Darouiche, 2004). The risk of infection after surgical implantation ranges from 1% and 7%, but is associated with considerable morbidity, repeated surgeries, and prolonged therapy (Anderson and Marchant, 2000). Infections associated with the insertion of vascular and urinary catheters are the most common serious complications of surgical implants (Darouiche, 2003). Furthermore, the mortality rate of an infected aortic graft can approach 40% (Anderson and Marchant, 2000), and infections of knee joint prostheses can, in up to 52% of cases, lead to ankylosis and, in up to 9% of the cases, to amputation (Gollwitzer et al., 2005). Prevention of such infections remains a priority (Darouiche, 2003).

A new strategy for preventing implant-associated infections involves coating the implants with a polymer that contains common antibiotics. Such approaches are currently in clinical trials (Darouiche, 2003; Gollwitzer et al., 2005). However, the rising problem of infections caused by multiply antibiotic-resistant bacteria, so-called superbugs, limits the value of this approach. In addition, the standard procedure for treating implant-associated infections, using high doses of antibiotics over a long period of time, might exacerbate this situation by contributing to selection of antibiotic-resistant bacteria with potential life-threatening complications for patients. The development of an implant coating with broad spectrum antimicrobial activity and one that has no relationship to common antibiotics would be highly advantageous.

Cationic antimicrobial peptides are among the most prominent antimicrobial substances produced by most complex organisms for local protection (e.g., of the skin and mucosal surfaces) against microbial infections (Harder et al., 2001). They have emerged as central components of the innate defenses of animals, insects, and plants, and peptides with activities against Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, and eukaryotic parasites have been identified (Harder et al., 2001). Indeed, the therapeutic potential of cationic antimicrobial peptides as alternatives to conventional antibiotics is currently being explored with synthetic peptides demonstrating efficacy in phase IIIa clinical trials (Hamill et al., 2008). The mechanisms of action of these peptides are complex and different from conventional antibiotics, and it has proven extraordinarily difficult to select resistant mutants to such peptides. The available evidence indicates that cationic antimicrobial peptides interact with bacterial surfaces to either permeabilize them or to translocate across the cytoplasmic membrane to attack cytoplasmic targets. Although the majority of studies of the cationic antimicrobial peptides have focused

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on free peptides in solution, it was previously demonstrated that the cationic lipopeptide polymyxin B could inhibit the growth of the Gram-negative bacterium *Escherichia coli* while covalently bound to an agarose bead (LaPorte et al., 1977). Subsequently, Haynie et al. (1995) demonstrated that surface-immobilized (tethered) cationic antimicrobial peptides had broad spectrum activity, including the ability to kill Gram-positive bacteria and yeast.

Thus, short-tethered cationic antimicrobial peptides appear to be excellent candidates for protecting surfaces against microbial growth, such as those of medical implants. Although many investigations of soluble antimicrobial peptides have served to establish the structure-activity relationships that dictate peptide antimicrobial activity and cytotoxicity, this is not the case for tethered antimicrobial peptides. Indeed, as immobilization of peptides to a surface would result in limitations to peptide mobility and thus the ability of peptides to enter into or translocate across membranes, it is imperative that structure-activity relationship investigations among tethered cationic antimicrobial peptides be established. Previously, we developed a high-throughput antimicrobial peptide activity screening assay utilizing *Pseudomonas aeruginosa* with a constitutively expressed luciferase (*luxCDABE*) gene cassette; however, this method was limited to free peptides in solution (Hilpert et al., 2005, 2006). Here we have adapted and made key modifications to this methodology to enable the identification of surface-bound peptides with antimicrobial activity using a high-throughput screening assay format. By creating a large library of peptides, we were able to investigate the influence of charged and hydrophobic residues on the antimicrobial activity of tethered peptides, as well as the influence of their positioning within the peptide sequence relative to the tethering surface. The resultant strategy will assist the development of peptidic antimicrobial surfaces that might exhibit certain advantages over those presently used in the clinic.

RESULTS

Methods Development

Peptides were produced by a variation of the SPOT synthesis method involving synthesis of peptides on a membrane support as described in detail previously (Frank, 1992; Hilpert et al., 2007). A variety of linkers were tested, and we eventually decided to use the CAPE linker strategy, designed for high stability by utilizing an ether bond (Kamradt and Volkmer-Engert, 2004). Spontaneous peptide release from the respective tethering surface of the CAPE-linked active peptide Bac2A and negative control peptide Tet000 was monitored by HPLC after 4 hr of incubation at 37°C in 100 mM Tris-HCl buffer (pH 7.5). No spontaneous peptide release was detected by HPLC using the CAPE linker, supporting the use of this linker strategy. By comparison, peptides were almost completely released after overnight treatment with ammonia gas.

Using the SPOT synthesis technology, the peptides Bac2A and variants of Bac2A were synthesized at a peptide density of 50 nmol/spot (Hilpert et al., 2005). The peptide Tet000, an inactive unrelated peptide (Hilpert et al., 2005), was synthesized and included as a negative control. Following cleavage of the side-chain protecting groups and an intense washing procedure, the peptide spots were punched out of the cellulose sheet and transferred into a standard 96-well microtiter plate suitable for luminescence measurements (one peptide spot per well). A luminescent *Pseudomonas aeruginosa* reporter strain, H1001, containing the luciferase reporter gene *fliC::luxCDABE* was employed. For this strain, bacterial luminescence is dependent on cellular energization and is therefore directly related to bacterial proliferation. Bacteria, glucose, and buffer were added, and luminescence monitored over time after peptide addition. The peptides Tet008 and Tet009 were clearly able to decrease the luminescence of *P. aeruginosa*, with only limited reduction of luminescence upon incubation with the inactive control peptide Tet000 (Table 1). Thus, the decreased luminescence noted for the active peptides was not a result of residual chemicals used during peptide synthesis. As described previously (Hilpert et al., 2005), it could be demonstrated that a nearly perfect correlation existed between bacterial luminescence and the residual colony counts of surviving bacteria after overnight incubation (Figure 1), confirming that the *lux* screening protocol could be used accurately for the assessment of tethered peptide antimicrobial activities.

Screening for Antimicrobial Activity

Cathelicidins are a family of variable, naturally occurring antimicrobial peptides that are grouped based on their common prepro sequences, even though the mature active peptide fragments tend to be structurally very different (Hancock and Sahl, 2006). To decrease any potential for bias, and permit screening for tethered peptides with enhanced antimicrobial activity, two very different cathelicidin peptides were chosen as starting points for making variant peptides. The 12 aa peptide Bac2A (RLARIV VIRVAR) (Wu and Hancock, 1999a), a linear variant of the naturally occurring cyclic peptide bactenecin (also called bovine dodecapeptide), is one of the smallest naturally occurring cationic antimicrobial peptides (Romeo et al., 1988) and is active in solution against Gram-positive and Gram-negative bacteria. The 13 aa extended peptide indolicidin (ILPWKWPWWPWRR) (Rozek et al., 2000), containing the highest proportion of Trp of any natural peptide (Zanetti et al., 1995), was also chosen, as an indolicidin derivative, MX-226, is currently in phase IIIa human clinical trials (Hamill et al., 2008).

The screening procedure was initially performed using a peptide density of 50 nmol/spot and/or 200 nmol/spot. At both peptide densities, the screen was repeated three times for each tethered peptide spot, resulting in three independent rounds of biological assays. Using a peptide concentration of 200 nmol/spot, 23 different sequences were discovered that showed inhibitory values of more than 90% at one or both peptide concentrations (Table 1), representing 17.2% of the total tested library of 122 peptides (other peptides screened appear in Table S1 available online). It was also noted that 9-, 12-, and 13-mer peptides were primarily represented among these highly active peptides. In general, as the inhibitory effect increased, the standard deviation decreased, indicating a higher confidence for higher values of inhibition. Illustrating this point, the peptide Tet009 showed a mean inhibition of *P. aeruginosa* luminescence of 84.7% ± 7.6% as compared with peptide Tet052 (44.6% ± 8.8% inhibition) and Bac2A (16.9% ± 15.7% inhibition). An exception to this correlation was found for proline-containing

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