Adding Selectivity to Antimicrobial Peptides: Rational Design of a Multidomain Peptide against *Pseudomonas* spp.

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Currently available antimicrobials exhibit broad killing with regard to bacterial genera and species. Indiscriminate killing of microbes by these conventional antibiotics can disrupt the ecological balance of the indigenous microbial flora, often resulting in negative clinical consequences. Species-specific antimicrobials capable of precisely targeting pathogenic bacteria without damaging benign microorganisms provide a means of avoiding this problem. In this communication, we report the successful creation of the first synthetic, target-specific antimicrobial peptide, G10KHc, via addition of a rationally designed *Pseudomonas*-specific targeting moiety (KH) to a generally killing peptide (novispirin G10). The resulting chimeric peptide showed enhanced bactericidal activity and faster killing kinetics against *Pseudomonas* spp. than G10 alone. The enhanced killing activities are due to increased binding and penetration of the outer membrane of *Pseudomonas* sp. cells. These properties were not observed in tests of untargeted bacterial species, and this specificity allowed G10KHc to selectively eliminate *Pseudomonas* spp. from mixed cultures. This work lays a foundation for generating target-specific “smart” antimicrobials to complement currently available conventional antibiotics.

Human mucosal surfaces are colonized by large numbers of bacteria. This indigenous microflora plays an important role in human health by providing nutrients, preventing colonization by pathogens, and stimulating immune functions of the host (3, 23, 24, 28, 29, 39). Environmental and biological factors may disrupt the normal flora and lead to mucosal microbial infections, several of which stand among the most prevalent diseases worldwide (20, 31). Because immune function at mucosal surfaces is limited, antibiotics have been the treatments of choice for mucosal infections. Current antibiotics have broad spectra of activity, killing benign or beneficial commensal bacteria as well as the pathogens they intend to target. This often leads to severe posttreatment complications (3, 19); for example, several opportunistic pathogens such as *Clostridium difficile*, *Candida albicans*, and *Staphylococcus aureus* take advantage of the increase in niche size after antibiotic treatment, often causing serious “antibiotic-associated” infections (14, 30). These problems, combined with the increasing emergence of pathogens that are resistant to conventional antibiotics, underscore the urgent need for new antimicrobials that are capable of targeting specific pathogens without harming the normal flora.

A few attempts have been made to achieve target-specific antimicrobial therapy by conjugating antibiotics to species-specific monoclonal antibodies or creating fusion proteins that combine bactericidal domains with bacterial recognition domains (26, 27). Neither method has yielded a satisfactory solution due to the low efficiency of chemical conjugation, instability of large proteins, or high cost of production. Small peptides, however, including numerous antimicrobial peptides,

can easily be synthesized in high yields. Antimicrobial peptides have emerged as attractive alternatives to conventional small-molecule antibiotics, mainly because of their ability to kill antibiotic-resistant pathogens (5, 8).

Echoing prior attempts at fusing specific targeting domains to antimicrobial compounds, the antimicrobial peptide nisin was found to contain a targeting domain in addition to its killing domain, which increased the potency of the peptide against its target bacteria (4). The presence of this targeting domain increased the accumulation of nisin molecules on the surfaces of the target bacteria, generating higher local peptide concentrations and providing a mechanism for the enhanced antimicrobial activity (1, 5, 16, 35). Based on these findings, we hypothesized that the addition of short targeting peptides to naturally derived broad-spectrum antimicrobial peptides would convert them into “smart” compounds with selective bactericidal activities. Here we report the successful design and synthesis of such a chimeric antimicrobial peptide that can specifically target *Pseudomonas* spp., including *P. mendocina* (2).

MATERIALS AND METHODS

Strains and growth conditions. Of the strains used in this study (Table 1), *Pseudomonas mendocina* PM5 (this study), *Pseudomonas fluorescens* 1088 (kindly provided by A. Hirsch, University of California at Los Angeles [UCLA]), *Pseudomonas aeruginosa* PAK (38), *Escherichia coli* W3110 (43), *Klebsiella pneumoniae* KAY2026 (36), and *Staphylococcus aureus* Newman (10) were grown at 37°C in Luria-Bertani broth medium (LB; Fisher) under aerobic conditions. *Streptococcus mutans* UA159 (7) was grown in Todd-Hewitt (TH; Fisher) broth medium at 37°C under anaerobic conditions.

Synthesis and purification of peptides. Potential targeting peptides (Table 1), novispirin G10 (KNLRRIRKGIHIKKYG), and selectively targeted antimicrobial peptides (STAMPs) G10KHc (KKHRKHKRKH-GGSGGS-KNLRRIRKGIHIKKYG) and G10KHn (KNLRRIRKGIHIKKYG-GGSGGS-KKHKRKH) were synthesized using Fast-Fmoc (9-fluorenylmethoxy carbonyl) solid-phase methodology (431A peptide synthesizer; Applied Biosciences) with double-coupling cycles. Peptides were cleaved from the resin with 95% trifluoroacetic acid with appropriate scavengers and purified to 90 to 95% by reverse-phase high-

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TABLE 1. Binding profiles of fluorescently labeled potential targeting peptides against bacteria

Peptide	Signal ^a						
	1088	PM5	PAK	KAY2026	W3110	UA159	Newman
KH (KKHRKHKHRKH)	+	++	+	—	—	+/-	+/-
PH-1 (KPVLPVLPVLPV)	—	—	—	—	—	+/-	+
LP-1 (VLRIIRIAVLRIIRIA)	—	+/-	—	—	—	+/-	+/-
S3L1-5 (WWYNWWQDW)	+	+	+	+	+	+	+
S3L1-10 (FIKDFIERF)	+/-	+/-	+/-	+/-	+/-	+	+
S3L1-18 (LIQDILNEL)	—	—	—	—	—	+/-	+/-
S3L1-25 (AMKHAMHRV)	—	—	—	—	—	—	+/-
S3L1-35 (AAYNAAQDA)	—	—	—	—	—	—	+/-

^a Semiquantitative scale: ++, majority of cells brightly stained; +, low-intensity fluorescent signal; +/-, very weak or diffuse fluorescent signal; —, no signal. Strains: 1088, *P. fluorescens*; PM5, *P. mendocina*; PAK, *P. aeruginosa*; KAY2026, *K. pneumoniae*; W3110, *E. coli*; UA159, *S. mutans*; Newman, *S. aureus*.

performance liquid chromatography (ACTA purifier; Amersham). Peptide molecular mass was determined by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

Fluorescent labeling of peptides and fluorescence microscopy. Before cleavage and purification, an aliquot of each peptide in this study was labeled with carboxyfluorescein (Sigma) as previously described (11). The resin was thoroughly washed to remove unconjugated dye, and the fluorescence intensity of the labeled peptides was measured fluorimetrically ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 520$ nm; VersaFluor; Bio-Rad) after peptide cleavage. To examine fluorescent peptide binding to bacteria, exponentially growing cells (optical density at 600 nm [OD₆₀₀] of 0.5) were first washed with phosphate-buffered saline (PBS), diluted 1:2 into PBS, and incubated at 25, 10, or 4°C. Peptide (16 μ M) was then added for 1 to 2 min. Cells were collected by centrifugation (5 min, 16,000 $\times$ g), washed repeatedly with PBS to remove unbound peptide, and resuspended in PBS. Binding was evaluated using bright-field and fluorescence microscopy (Nikon E400) at $\times 40$ magnification. Digital images were collected (SPOT RT; Diagnostic Instruments) using factory-supplied settings for bright-field and green fluorescence.

Determination of antimicrobial activity. Peptide antimicrobial activity was determined by using a standard MIC assay in Mueller-Hinton (MH) broth (21) for all organisms except *S. mutans*, for which an MIC assay with TH broth (instead of MH) was utilized (25). Briefly, exponentially growing bacterial cells were diluted to $\sim 10^5$ CFU/ml in MH or TH and placed into 96-well plates. Peptides were serially diluted and added to the bacteria. The MIC was determined by identifying the concentration of peptide that completely inhibited bacterial growth after ~ 24 h incubation.

Determination of bactericidal kinetics. Bacteria were grown to log phase and diluted to $\sim 10^5$ CFU/ml in growth medium. Under aerobic conditions, peptides were added to the cell suspension and incubated at 25°C (4 and 10°C for temperature dependence experiments). At each time point, 10 μ l of cell suspension was removed, rescued by dilution into 500 μ l growth medium, and kept on ice until plating. For plating, 20 to 500 μ l of rescued cells was spread on growth medium agar plates and colonies were counted after overnight incubation at 37°C under aerobic (*P. mendocina* and *E. coli*) or anaerobic (*S. mutans*) conditions. We considered 60 CFU/ml the detection limit for this assay.

For mixed-culture bactericidal kinetics, approximately 3×10^5 to 5×10^5 CFU/ml of *P. mendocina* and *S. mutans* was coinoculated in growth medium with 12 μ M peptide at room temperature as in the monoculture killing kinetics assay. Survivors were recovered as described above and spread on both LB and TH plates. The two bacterial species were differentiated by their opposite oxygen requirements: rescued *P. mendocina* cells were counted from LB plates after overnight incubation under aerobic conditions and *S. mutans* cells were counted from TH plates after overnight incubation under anaerobic conditions.

For growth retardation experiments to examine the sustained killing activity of antimicrobial peptides, $\sim 5 \times 10^6$ CFU/ml bacteria were exposed 9 μ M peptide for 15 min at 25°C. Cells were then washed several times and resuspended in 1 $\times$ PBS, normalized to an OD₆₀₀ value of 0.05 to 0.1, and added to a 96-well plate. Cells were incubated at 37°C under aerobic (*E. coli* and *P. mendocina*) or anaerobic (*S. mutans*) conditions and monitored for growth by measuring the OD₆₀₀ with a microplate spectrophotometer (Benchmark Plus; Bio-Rad).

Evaluation of outer membrane integrity. The outer membrane integrity after peptide addition was evaluated with the dye N-phenyl-1-naphthylamine (NPN), as described previously (17, 33). NPN was added to $\sim 10^5$ CFU/ml of log-phase cells to a final concentration of 10 μ M. The mixture was transferred to a 1-ml plastic cuvette and allowed to equilibrate until the fluorescence stabilized (approximately 3 to 5

min). Peptide was added to the cuvette and the increase in fluorescence was measured ($\lambda_{\text{ex}} = 337$ nm, $\lambda_{\text{em}} = 426$ nm; VersaFluor). Loss of outer membrane integrity was quantified as the increase in fluorescence after peptide addition at designated time points (after subtraction of background fluorescence).

Evaluation of inner membrane integrity. Bacterial inner membrane integrity after peptide treatment was measured using 3,5-dipropylthiadicarbocyanine iodide (diSC₃₋₅) (Molecular Probes) as previously described (40, 42). Briefly, spheroplasts were first prepared from gram-negative cells as previously described (22), adjusted to 5×10^4 to 10^5 CFU/ml, and preequilibrated with 0.4 μ M diSC₃₋₅ until dye uptake ceased (indicated by a stable reduction in fluorescence; about 1 h); 100 mM KCl was added to equilibrate K⁺ levels prior to the addition of peptides. This concentration of KCl has no effect on peptide activity (data not shown). Fluorescence was measured in a 96-well plate format using a Spectra-Max Gemini XS plate reader ($\lambda_{\text{ex}} = 622$ nm, $\lambda_{\text{em}} = 670$ nm; Molecular Devices, Sunnyvale, CA). Loss of membrane integrity was defined as the increase in fluorescence after peptide addition and subtraction of background fluorescence.

RESULTS

Design and synthesis of targeted antimicrobial peptide G10KHC. Working from the hypothesis that addition of a species-specific targeting domain to a broad-spectrum antimicrobial peptide would result in a target-specific antimicrobial agent, a linear peptide was designed that combined the antimicrobial peptide novispirin G10 (KNLRRRIIRKGIHIKKYG), via an appropriate linker region, with a novel targeting domain. We chose novispirin G10 as the killing peptide because it is relatively short, has low toxicity, and shows similar efficacies against a wide range of gram-positive and gram-negative bacteria (32, 37, 41).

Although a monoclonal antibody and a “minibody” derived from the variable regions of an antibody were potential targeting domains, we found them unstable and difficult to produce in large quantities when fused to G10 (W. Shi, U.S. patent application 20030143234). As an alternative approach, we focused on a set of short (8 to 12 amino acids) fluorescently labeled peptides that varied widely in their chemical and physical properties (such as hydrophobicity and charge). The peptides were synthesized and visually screened by fluorescence microscopy for the ability to bind to different bacterial species (Table 1). We were able to identify one peptide (KKHRKHKHRKH), designated KH, which exhibited strong binding to various *Pseudomonas* spp. (*P. mendocina* was used as a representative species in this study), but little or no binding to *E. coli* and *S. mutans* (Table 1 and Fig. 1) (R. Eckert, D. K. Yarbrough, J. He, F. Qi, and W. Shi, unpublished data). KH did not show any antimicrobial activity in an MIC assay (Table 2). KH was thus chosen as the targeting domain against *P. mendocina*.

FIG. 1. Binding of KH to bacteria at 25°C. Bright-field (left column) and fluorescence (right column) images of the same field were acquired after each bacterium, *P. mendocina* (Pm), *E. coli* (Ec), and *S. mutans* (Sm), was incubated with fluorescently labeled peptide solution (16 μ M) for 1 min and washed with PBS. Cells with peptide bound are stained green. Scale bars, 10 μ m.

To ensure the proper function of both the targeting peptide (KH) and killing peptide (G10), we incorporated a linker region between these two functional domains. Linkers of different lengths containing various combinations of glycine and serine residues were devised and tested. Peptides containing the linker sequence GSGGS showed the least effect on both the antimicrobial activity of G10 and the targeting ability of KH (R. Eckert, D. K. Yarbrough, J. He, F. Qi, and W. Shi, unpublished observations).

To construct the chimeric peptide, two variants of the full G10-linker-KH combination, KH-GSGGS-G10 (called G10KHc in this study) and G10-GSGGS-KH (designated G10KHn), were synthesized and their antimicrobial activities were compared with that of G10. As shown in Table 2, addition of G10-GSGGS at the N terminus of KH (G10KHn) reduced the antimicrobial activity of this peptide relative to that of G10 alone, while addition of GSGGS-G10 at the C terminus of KH (G10KHc) caused little or no reduction in antimicrobial activity. This peptide, G10KHc, was selected for further analysis as described below.

Characterization of G10KHc for its specific anti-*P. mendocina* activity. The MICs of G10 and G10KHc against *P. mendocina*, *E. coli*, and *S. mutans* were similar (Table 2). This is likely due to the fact that the assay to determine MICs relies on evaluations of culture growth after 18 to 24 h (see Materials and

Methods): given the fact that G10KHc and G10 both contain the same bactericidal domain, it was not surprising that they would show similar activities over long periods of time. Because this assay only measures the concentration of each peptide that is required to maximally inhibit culture growth, differences in the rate of killing between the two peptides may be obscured. We anticipated that the targeting ability of KH would increase the rate with which G10KHc kills *P. mendocina*, relative to that of G10 alone, and that this effect would not extend to other species. Therefore, the kinetics and selectivity of G10 and G10KHc activity were investigated further.

To determine whether G10KHc has enhanced and specific anti-*P. mendocina* activity, we performed the following killing kinetic experiments on three test strains (*P. mendocina*, *E. coli*, and *S. mutans*). A conspicuous difference in killing rate was observed between G10KHc and G10 against *P. mendocina* (Fig. 2a). After 2 min of peptide treatment, 3 μ M G10KHc killed over 100-fold more *P. mendocina* than did the same molar concentration of G10. An even greater difference in survivors was seen at 6 μ M: G10KHc eliminated nearly 1,000-fold more *P. mendocina* cells than G10 after only 1 min of treatment. After 5 min of treatment, G10KHc at both concentrations reduced *P. mendocina* to the detection limit for this assay, while 3 and 6 μ M G10 failed to reach this level of activity even after continuing treatment for 2 h.

Consistent with our hypothesis, the lengths of treatment required for G10 and G10KHc to kill *E. coli* and *S. mutans* were similar (Fig. 2b and c). Comparing killing by G10 and G10KHc at a single time point (2 min), the enhanced killing ability of G10KHc against *P. mendocina* was clear (Fig. 2a and c); while G10KHc and G10 alone showed similar activities against *S. mutans* and *E. coli*, G10KHc exhibited dramatically increased killing ability against *P. mendocina* relative to G10. Taken together, these results demonstrate that G10KHc kills *P. mendocina* at a much faster rate than G10 alone. More importantly, these data also show that other bacterial species are not subject to this effect. Notably, the differences in the antimicrobial activity of G10KHc against the tested species correlated well with the binding abilities of KH shown in Fig. 1.

Selective inhibition of *P. mendocina* growth was also observed when we investigated if G10KHc could retard culture growth after brief exposure, followed by stringent washing to remove unbound peptide. In these experiments, cultures were treated with G10KHc or G10 for a brief period of time, washed, and then transferred to rich medium for growth. The results (Fig. 3) indicated that, even after washing, G10KHc was still able to retard *P. mendocina* growth for 180 min, while *E. coli* and *S. mutans* were unaffected, similar to the results in Fig. 2.

TABLE 2. MICs of peptides novispirin G10, G10KHn, G10KHc, and KH

Species and strain	Avg MIC ^a (μ M) $\pm$ SD			
	G10	G10KHc	G10KHn	KH
<i>Pseudomonas mendocina</i> PM5	2.6 $\pm$ 1.34	2.8 $\pm$ 1.63	23 $\pm$ 6.78	>25
<i>Escherichia coli</i> W3110	3.5 $\pm$ 2.67	5.4 $\pm$ 1.11	>25	>25
<i>Streptococcus mutans</i> UA159	10.5 $\pm$ 3.51	10.5 $\pm$ 3.51	>25	>25

^a MICs represent averages of at least three independent experiments with standard deviations.

FIG. 2. Killing kinetics of G10 and G10KHc. All tested bacteria, (a) *P. mendocina* (Pm), (b) *S. mutans* (Sm), and (c) *E. coli* (Ec), were treated with peptide solutions (specified in the figure) for 30 seconds to 2 h and survivors were plated. Data points represent averages of three independent experiments.

Having established that G10KHc had improved killing kinetics against *P. mendocina* in monoculture conditions, we next sought to determine whether G10KHc was able to exhibit selectivity for *P. mendocina* in a mixed species environment.

FIG. 3. Sustained killing of G10KHc against targeted bacteria. *P. mendocina* (Pm), *E. coli* (Ec), and *S. mutans* (Sm) were exposed to no peptide (solid triangles) or 9 μM G10 (diamonds) or G10KHc (solid squares) for 15 min, thoroughly washed with PBS, and regrown in rich medium. Regrowth was measured by OD₆₀₀ and plotted through 180 min. Data points represent averages of three independent experiments.

Figure 4 shows a representative experiment: *S. mutans* and *P. mendocina* cells were mixed in a 1:1 ratio, and the resulting mixtures were treated with G10 or G10KHc. Consistent with the activities observed in monocultures, G10KHc selectively killed *P. mendocina* in the *S. mutans*-*P. mendocina* mixed cultures (Fig. 4). After 1 to 2 min of G10KHc treatment, the ratio of recovered *P. mendocina* to *S. mutans* cells was markedly smaller than the ratio of survivors found in G10-treated cultures. Selective killing activity of G10KHc against *P.*

FIG. 4. Killing selectivity of G10 and G10KHc. A 1:1 mixture of *S. mutans* (Sm) and *P. mendocina* (Pm) was treated with 12 μ M G10KHc or G10 for 30 seconds to 5 min, and survivors were quantitated. The change in relative ratio of *P. mendocina* to *S. mutans* (Pm/Sm) after the G10KHc (open diamonds) or G10 (solid squares) treatment is shown. Data points represent averages of three independent experiments.

mendocina was also observed in mixed cultures with other bacterial species (data not shown). G10KHc is therefore capable of preferentially eliminating the target species from within a mixed bacterial culture.

Enhanced killing activity of G10KHc against *P. mendocina* is conferred by its targeting specificity. The data presented above clearly show that G10KHc selectively kills *P. mendocina* cells. Given the known specificity of the KH peptide for *P. mendocina* (Fig. 1), it is likely that this selectively enhanced activity of G10KHc emerges from a mechanism in which the KH moiety causes selective accumulation of G10KHc on the *P. mendocina* cell surface. In this case, the concomitant increase in the local

concentration of the G10 moiety then leads to the increase in antimicrobial activity. Alternatively, it is possible that the addition of KH may have enhanced the intrinsic ability of the G10 moiety to penetrate *P. mendocina* membranes, independent of binding to the cell surface. Mechanistic studies were undertaken in order to distinguish between these two possibilities.

Binding assays against *P. mendocina*, *S. mutans*, and *E. coli* cells were performed using fluorescently labeled G10 and G10KHc. As shown in Fig. 5, fluorescently labeled G10KHc stained *P. mendocina* cells brightly, but exhibited very limited binding to *E. coli* or *S. mutans* cells. In contrast, G10 did not bind significantly to any of the three bacterial species tested during the short incubation period. Peptide fluorescence was measured ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 520$ nm) to ensure that this result was not due to differences in the efficiency of fluorescein labeling between G10 and G10KHc: G10 and G10KHc showed similar fluorescence intensities at the same molar concentration (data not shown). Notably, the binding profile of G10KHc (Fig. 5) was consistent with the binding profile of KH (Fig. 1), suggesting that the targeting ability of G10KHc was derived from the binding activity of the KH moiety. Additionally, detailed quantitative binding experiments have been carried out, showing that levels of peptide binding at equilibrium correlate well with the results shown in Fig. 1 and 5 (R. Eckert, D. K. Yarbrough, J. He, F. Qi, and W. Shi, unpublished data).

To obtain further evidence that the enhanced activity of G10KHc against *P. mendocina* is due to an increase in targeting specificity rather than a generally enhanced killing ability, we performed bactericidal kinetic studies similar to those in Fig. 2 at 4, 10, and 25°C. Previous reports describing the tem-

FIG. 5. Binding of G10 and G10KHc to bacteria. Brightfield (left column) and fluorescence (right column) images of the same field were acquired after each bacterium, *P. mendocina* (Pm), *E. coli* (Ec), and *S. mutans* (Sm), was incubated with fluorescently labeled peptide solution (16 μ M) for 1 min and washed with PBS. Cells with peptide bound are stained green. Scale bars, 10 μ m.

FIG. 6. Effect of temperature on bioactivities of G10KHc. (a) Bactericidal activity of G10KHc was evaluated at 4, 10, and 25°C against *P. mendocina* from 30 seconds to 2 h exposure of the peptide (symbols are defined in the figure). Data points represent averages of three independent experiments. (b) Bright-field (left column) and fluorescence (right column) images of the binding of G10KHc to *P. mendocina* (Pm) or *S. mutans* (Sm) at 4 or 25°C. Scale bars, 10 μ m.

perature dependence of antimicrobial peptide activity suggest that low temperature does not affect the primary binding step but does inhibit or slow subsequent events due to a reduction in postbinding peptide-lipid interactions (16). As shown in Fig. 6, at 4°C, G10KHc no longer exhibited antimicrobial activities against *P. mendocina* (Fig. 6a); however it retained its strong targeting ability, binding to *P. mendocina* but not to *S. mutans*. Similar binding behavior, along with restored antimicrobial activities, was observed at 25°C (Fig. 6b). These data demonstrate that G10KHc recognizes *P. mendocina* during the primary binding event, likely mediated by the KH targeting domain. Additionally, the uncoupling of the targeting and killing ability of G10KHc by low temperature is consistent with the idea that the enhanced activity of G10KHc against *P. mendocina* is a result of an enhanced species-specific binding activity that is independent of the membrane-disrupting activity of the G10 moiety.

Enhanced G10KHc activity against *P. mendocina* is due to increased outer membrane permeability. Although the above data demonstrated that G10KHc preferentially accumulates on the surfaces of *P. mendocina* cells, the mechanism by which G10KHc kills these cells remained to be elucidated. Antimi-

crobial peptides generally act on bacteria by destabilizing or disrupting the cytoplasmic membrane (5, 35). In gram-negative bacteria, the outer membrane presents an additional barrier to attack by these peptides (35). To gain a better understanding of how G10KHc kills *P. mendocina*, we separately examined the effect of this peptide on the integrity of the inner and outer membranes.

To compare the ability of G10KHc and G10 to destabilize and cross the outer membranes of *P. mendocina* and other bacteria, we used the conditional fluorescent dye *N*-phenyl-1-naphthylamine as previously described (17, 33, 34). NPN is completely quenched in aqueous environments, whereas in a hydrophobic environment, such as the interior of cellular membranes, it fluoresces strongly. Thus, as the outer membrane is destabilized, more NPN is able to intercalate into the inner membrane and fluoresce.

Figure 7a shows the results from one of these experiments, in which we compared outer membrane disruption in *P. mendocina* versus *E. coli*. At 5 to 10 seconds and at 30 seconds after peptide addition, NPN fluorescence from samples containing G10KHc-treated *P. mendocina* culture was fivefold higher than that of parallel samples treated with G10. In contrast, no clear

FIG. 7. Effect of G10 and G10KHc on integrity of outer and inner membranes. Outer membrane integrity (a) was measured by the increase in NPN fluorescence units (FU) at 5 or 30 seconds after 3 μ M G10 (open bars) or G10KHc (shaded bars) addition. Inner membrane integrity (b) was measured as the increase in diSC₃-5 fluorescence 1 min after addition of 20 μ M G10 (open bars) or G10KHc (shaded bars) to *P. mendocina* (Pm) and *E. coli* (Ec). See the text for detailed experimental protocols.

difference was observed in *E. coli* cells at either time point. Based on these results, G10KHc shows a conspicuous increase in the degree of outer membrane destabilization in *P. mendocina* relative to that conferred by G10. The absence of any difference between the activities of G10KHc and G10 toward the outer membrane of *E. coli* is also fully consistent with the binding data presented in Fig. 1 and 5.

The activity of G10 and G10KHc at the inner membrane of *P. mendocina* and *E. coli* cells was investigated using diSC₃-5, a dye sensitive to disruption of the transmembrane electrostatic potential (40, 42). diSC₃-5 remains quenched within cells, but fluoresces brightly upon destabilization of the membrane. Spheroplasts of *P. mendocina* and *E. coli* were equilibrated with diSC₃-5 and then exposed to peptide. The subsequent change in fluorescence was recorded and, as seen in Fig. 7b, we observed no difference in the rate of fluorescence increase as a result of addition of G10 or G10KHc to either *P. mendocina* or *E. coli*. Thus, G10 and G10KHc were similarly effective at disrupting the inner membrane of *P. mendocina* and *E. coli*. Together, the results of the outer and inner membrane permeability assays show that the enhancement seen in the antimicrobial activity of G10KHc against *P. mendocina* is primarily a function of its ability to act at the outer membrane.

DISCUSSION

The majority of antimicrobial compounds in clinical use have broad killing spectra. While these antibiotics have the advantage that they can be used against bacterial infections without precise diagnosis, they also kill benign and beneficial commensal bacteria, thus disrupting the normal flora and creating numerous adverse side effects. Many of these postantibiotic complications could be avoided through the use of pathogen-specific antimicrobials. As methods are developed that allow the rapid and precise diagnosis of microbial infections, such a targeted antimicrobial therapy will become more and more desirable.

Since 1998, our laboratory has been actively developing target-specific antimicrobials. Our initial attempts focused on conjugating antimicrobial compounds to species-specific monoclonal antibodies and on creating fusion proteins between monoclonal antibodies and antimicrobial peptides (W. Shi, U.S. patent application 20030143234). In addition to our

efforts, Qiu et al. have recently reported the creation of 70-kDa recombinant target-specific antimicrobial proteins consisting of bactericidal proteins genetically fused to bacterial pheromones (26, 27). While all of these approaches created molecules that specifically killed their target bacteria, they are subject to significant drawbacks, such as difficulties in chemical conjugation that limit in vitro synthetic routes and problems with large-scale fermentation and purification that limit the utility of heterologous expression techniques. Thus, these approaches have yet to yield a clinically useful product.

Here, we successfully designed and tested a synthetic 36-amino-acid peptide (G10KHc) which exhibited strong antimicrobial activity as well as selectivity against a single target bacterium. Given the simplicity and universality of this approach, combined with the ease and scalability of in vitro peptide synthesis, we believe that the method described here could turn targeted antimicrobial therapy into a reality.

G10KHc exhibits a high degree of specificity for the bacterium *P. mendocina*, binding tightly to the surface of this species and rapidly and selectively killing *P. mendocina* cells even in the presence of other bacterial species (Fig. 2 to 4). We achieved these enhanced activities by fusing a preselected targeting peptide to a broad-spectrum antimicrobial peptide. Although we cannot completely rule out the possibility that the addition of KH to G10 simply created a new antimicrobial with coincidentally better activity against this bacterium, the data presented strongly suggest that the specificity of G10KHc against *P. mendocina* results from an independent activity of the KH domain.

Our results support a model in which the targeting domain (KH) and killing domain (G10) of G10KHc function separately: the targeting domain of G10KHc selectively delivers the peptide to the *P. mendocina* surface and the killing domain then rapidly disrupts the outer membrane, increasing its ability to access the cytoplasmic membrane and cause cell death. The combination of these otherwise independent processes results in large overall enhancements in killing kinetics and selectivity. The ability of the targeting domain to bind tightly to the bacterium is also likely to be responsible for the persistence of antimicrobial activity after rigorous washing (Fig. 3). The idea of multiple domains in short peptides is further supported by recent studies of plantaricin, a naturally occurring peptide from *Lactobacillus plantarum*, which was found to contain functionally and structurally independent pheromone and antimicrobial domains within its short linear sequence (9, 13, 15).

It is clear from the data presented that the addition of the targeting domain to G10KHc provided an obvious increase in cell surface binding, outer membrane permeability, and bactericidal kinetics compared to unmodified G10. These increased activities were limited to the species targeted by KH (Table 1), including the *Pseudomonas* spp. *P. aeruginosa* and *P. fluorescens*, the details of which will be published in future reports (R. Eckert, D. K. Yarbrough, J. He, F. Qi, E. P. Greenberg, and W. Shi, unpublished observations). Therefore, we believe that our goal of constructing a chimeric, selective antimicrobial was achieved and that it is possible to improve the selectivity of antimicrobial peptides through the addition of a peptide targeting domain.

The present work provides the basis for technology in which target-specific antimicrobial peptides (STAMPs) could be gener-

ated against any desired bacterium. We envision that a general STAMP would consist of a killing peptide domain, a linker peptide domain, and a targeting peptide domain as in our prototype STAMP, G10KHc. It is worthwhile to note that, in addition to G10KHc, we have used this approach to create several additional target-specific antimicrobials (unpublished data), further supporting the multidomain hypothesis and illustrating the broad applicability of this method. With vast numbers of antimicrobial peptides available, identifying peptides with activity against a chosen bacterium is not difficult (5, 6, 12).

Problems arise, however, in finding a targeting peptide that is both selective and short. Several approaches were taken during our initial studies, including monoclonal antibodies and truncated "minibodies," none of which provided a functional peptide with the desired combination of small size and high selectivity. Recently, great strides were made toward species-specific therapeutics by utilizing bacterial pheromones as targeting peptides for a large antimicrobial protein (26, 27); however, the small number of bacterial species for which species-specific pheromones are known or characterized limits this interesting option.

The KH peptide was developed by screening a small set of peptides with vastly different chemical and physical properties. We have recently expanded this approach by developing a series of small, rationally designed peptide libraries from which additional binding peptides can be selected (D. K. Yarbrough, R. Eckert, J. He, F. Qi, and W. Shi, submitted for publication), providing us with an efficient and productive approach to the rapid screening and discovery of targeting peptides. Because an effective linker domain is required to ensure the activity of both targeting and killing domains, the realization of optimal activity will likely involve screening of several linker sequences. We envision that most STAMPs will ultimately contain fewer than 40 amino acids, allowing rapid and robust chemical synthesis *in vitro*.

STAMP technology has several attractive features and a promising potential for further development. G10KHc, as our prototype STAMP, clearly displayed greatly enhanced killing speed and selectivity, as well as a sustained killing ability. This latter trait could be useful in future applications (including STAMPs) to simplify the way drugs are delivered to any number of target cells *in vivo* (as well as reducing the amount of therapeutic required). Since STAMPs employ membrane-active antimicrobial peptides rather than using protein targets for bactericidal effects, the emergence of bacterial resistance will be minimized. Finally, the ease of combinatorial peptide chemistry could allow multiple targeting domains per STAMP, enabling the design of antimicrobials that are custom tailored for very specific subsets of target bacterial species. This idea is particularly appealing, considering that some chronic infections are caused by multiple microorganisms rather than a single species (18). At the same time, exceptionally resilient species could be targeted with a combination of antimicrobial peptides present on one STAMP molecule.

As we gain a greater understanding of microbial communities and continue to improve diagnostic speed and accuracy, an adaptable platform for creating target-specific antimicrobial therapeutics will become increasingly desirable. We believe that the STAMP technology provides a system that will allow (almost) unlimited combinations of characterized antimicrobial peptides and targeting peptides to rapidly generate STAMPs with high specificity and potency.

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