Translocating Proline-Rich Peptides from the Antimicrobial Peptide Bactenecin 7[†]

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ABSTRACT: The intracellular delivery of most peptides, proteins, and nucleotides to the cytoplasm and nucleus is impeded by the cell membrane. To allow simplified, noninvasive delivery of attached cargo, cell-permeant peptides that are either highly cationic or hydrophobic have been utilized. Because cell-permeable peptides share half of the structural features of antimicrobial peptides containing clusters of charge and hydrophobic residues, we have explored antimicrobial peptides as templates for designing cell-permeant peptides. We prepared synthetic fragments of Bac 7, an antimicrobial peptide with four 14-residue repeats from the bactenecin family. The dual functions of cell permeability and antimicrobial activity of Bac 7 were colocalized at the N-terminal 24 residues of Bac 7. In general, long fragments of Bac_{1-24} containing both regions were bactericidal and cell-permeable, whereas short fragments with only a cationic or hydrophobic region were cell-permeant without the attendant microbicidal activity when measured in a fluorescence quantitation assay and by confocal microscopy. In addition, the highly cationic fragments were capable of traversing the cell membrane and residing within the nucleus. A common characteristic shared by the cell-permeant Bac_{1-24} fragments, irrespective of their number of charged cationic amino acids, is their high proline content. A 10-residue proline-rich peptide with two arginine residues was capable of delivering a noncovalently linked protein into cells. Thus, the proline-rich peptides represent a potentially new class of cell-permeant peptides for intracellular delivery of protein cargo. Furthermore, our results suggest that antimicrobial peptides may represent a rich source of templates for designing cell-permeant peptides.

The identification of thousands of genes and proteins by the recent advances in genomics and proteomics has led to a need for a rapid method to facilitate their translocation across cell membranes for target validation and functional studies. Since most cell membranes are generally impermeable to peptides, peptidomimetics, proteins, and nucleotides, synthetic cell-permeant or translocating peptides have been developed to meet this demand and complement traditional delivery methods such as microinjection and electroporation. Cell-permeant, or translocating, peptides are characterized by their ability to breach the cell membrane and deliver attached cargoes without causing lethal membrane disruption (reviewed in ref *1*). Two major types of cell-permeant peptides have been identified. The first type is highly cationic with six or more charged amino acids, Lys or Arg, clustered in close proximity; they include HIV-1 Tat peptide (*2*), penetratin (*3*), the chimeric transportan (*4*), and heat shock protein Hsp70 (*5*). The second type is hydrophobic, and prominent examples include peptides based on the H-region of protein signal sequences (*6*, *7*). These cell-permeant peptides have proven to be valuable in the delivery of biologically active cargo to the cytoplasm and nucleus. However, limitations in cytotoxicity (*8*, *9*) and difficulty in laboratory handling (reviewed in ref *7*) associated with the use of these peptides have also been reported.

Interestingly, both cationic and hydrophobic cell-permeant peptides share half of the structural features with the diverse family of cationic antimicrobial peptides that contain in their primary or secondary structures both segregated charged and hydrophobic regions (*10*−*12*). These structural requirements are important for their membrane permeability, or pore-forming, functions that lead to the microbicidal mechanisms (*13*). Although the membranolytic property of antimicrobials will limit their usage as cell-permeant peptides, we reasoned that fragments of antimicrobial peptides containing only the charged or hydrophobic regions may possess membrane-permeant activity without the attendant bactericidal activity. This rationale has prompted us to examine antimicrobials as templates for designing new cell-permeant peptides useful for intracellular delivery.

Our initial effort has focused on the Pro/Arg- (PR-)[1] rich family of antimicrobials that include Bac 7, whose antimicrobial activity is due to its ability to inhibit the intracellular protein synthesis machinery (*14*, *15*), rather than to create pores in the cell surface. Bac 7, a 59-residue protein (Figure 1), belongs to the bactenecin family (*16*, *17*) that also includes the closely related PR-rich member Bac 5. These PR-rich peptides contain highly cationic charged amino acids

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[1] Abbreviations: Bac, bactenecin; HIV, human immunodeficiency virus; PR-rich, proline and arginine rich; RP-HPLC, reverse-phase high-pressure liquid chromatography; MIC, minimal inhibitory concentration; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CD, circular dichroism; PPII, polyproline type II.

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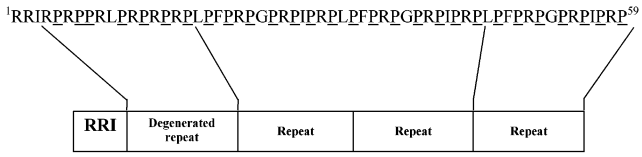

FIGURE 1: Amino acid sequence of full-length bovine Bac 7. The frequently occurring proline residues are underlined. Bac 7 is divided into five distinct regions: a charged cap, a degenerated repeat, and three copies of a 14-residue repeat.

at their N-termini and tandemly repeating sequences at their C-termini. The repeating 14-residue sequence found in Bac 7 provides an advantage for preparing synthetic fragments to dissect its structure−activity relationships.

The bactenecins, processed from their precursor cathelicidins, are located with other antimicrobial peptides in large granules within polymorphonuclear neutrophils of ruminants. Upon microbial infection, these granules fuse with phagosomes that contain engulfed microorganisms. The PR-rich peptides are then released into the phagosome to act upon the invading microorganisms and effectively kill the microbes (*18*, *19*). Our interest in studying Bac 7 is also enhanced by other members of the PR-rich peptide antimicrobials including PR-39 (*20*−*23*), abaecin (*24*), and apidaecin (*25*, *26*) that may have the ability to translocate through the microbial membrane to target intracellular proteins.

We report here a structure−activity study of the PR-rich antimicrobial peptide Bac 7 that explores its suitability as a template for designing new cell-permeant peptides. We dissected the dual functions of Bac 7 with truncated peptide fragments and were able to dissociate the translocating from the antimicrobial activity. Significantly, we found that all shortened Bac 7 fragments, irrespective of their charge or hydrophobic content, were cell-permeant and devoid of antimicrobial activity. These cell-permeant peptides contained the common Pro-Xaa motif. Our results suggest that Pro-rich peptides represent a new class of naturally occurring cell-permeant peptides that are soluble in aqueous solutions. Furthermore, antimicrobial peptides may represent a rich source of templates for designing novel cell-permeant peptides.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Fluorescent Labeling. Peptides were synthesized either manually or on an automated CS-Bio peptide synthesizer using Fmoc chemistry. All amino acids were supplied by Chem-Impex Int. (Wood Dale, IL). Fluorescent labeling was performed with the peptide attached to the solid support using 3 equiv of 5- (and 6-) carboxyfluorescein succinimidyl ester (Molecular Probes, Eugene, OR) in dimethylformamide for 2 h. The fluoresceinated peptides were then cleaved from the resin using a solution of trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5 v/v/v) for 3 h. All procedures involving fluoresceinated peptides were performed under dark conditions away from direct light. Peptides were separated from the resin by filtration and precipitated in diethyl ether before being dissolved in water or 60% aqueous acetonitrile. The crude peptides were purified by preparative RP-HPLC on a Waters 600 system using a C_{18} Vydac column. Products were confirmed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) using a PerSeptive Biosystems Voyager instrument. Measurements were taken in the linear mode with α-cyano-4 hydroxycinnamic acid as the matrix. Product purity (>95%) was confirmed using an analytical RP-HPLC with a C_{18} Vydac column installed in a Shimadzu LC system.

Cells and Cell Culture. RAW 264.7 murine monocytes were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were routinely maintained in DME medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA), 2 mM glutamine (Gibco, New York), 50 units/mL penicillin, and 50 mg of streptomycin (GIBCO-BRL, Rockville, MD). The cells were incubated at 37 °C in an atmosphere containing 5% CO_2, and cultures were replenished with fresh complete culture medium and reseeded twice weekly.

Antimicrobial Assay. A sensitive and reproducible two-stage radial diffusion assay described by Lehrer et al. (*27*) was employed for testing the antimicrobial activity of peptides. Three organisms obtained from the ATCC were used for these assays: Gram-negative *Escherichia coli* (ATCC 25922), Gram-positive *Staphylococcus aureus* (ATCC 29213), and the fungi *Candida albicans* (ATCC 37092). The microbes were incubated in trypticase soy broth (TSB) that had been prepared in doubly distilled water and sterilized by autoclave. The test organism was then mixed with a molten underlay gel solution and poured into 10 × 10 cm Petri dishes. The underlay gel solution contained 10 mM sodium phosphate buffer, 0.03% TSB, and 0.02% Tween 20. Wells 3 mm in diameter were punched into the underlay, and 5 μL aliquots of serial half-log dilutions of peptides were added to each well. The dishes were incubated at 37 °C for 3 h to allow the test peptides to diffuse into the underlay gel. The gels were then overlaid with 1% agarose containing 6% TSB. The diameter of the clear zone surrounding the wells was measured under a microscope after incubation at 37 °C for 16−24 h. Antimicrobial activity is expressed as minimal inhibitory concentration (MIC), which was determined from the *x*-intercepts of the dose−response curves.

Fluorescence Quantitation Assay. The quantitation assay was routinely performed under dark conditions away from direct light. Cells (3×10^5) in 150 μL were added to each well of a 96-well flat-bottomed, black with clear bottom plate (Corning, New York). All fluoresceinated peptides were dissolved in phosphate-buffered saline (PBS, pH 7.3) and the concentrations adjusted on the basis of the fluorescent intensity. The fluoresceinated peptides were added to wells in 50 μL aliquots with concentrations varying from 0.5 to 20 μM/well. Peptides and cells were incubated for 30 min at 37 °C in 5% CO_2. After the incubation, cells were centrifuged for 20 min at 2000 rpm, washed three times with PBS, resuspended in 200 μL of PBS, and lysed after three freeze−thaw cycles. Following a second centrifugation (20 min, 2000 rpm), 50 μL aliquots of supernatant were transferred to a new plate, and the fluorescence was quantitated using a SpectraMAX Gemini XS fluorescence spectrophotometer (Molecular Devices, CA) and SOFTmax PRO software (Molecular Devices). Fluorescence was measured with excitation set at 494 nm, emission at 525 nm, and cutoff at 515 nm.

For incubation in the presence of sodium azide, cells were preincubated in medium containing 0.5% sodium azide for 30 min prior to the addition of peptide. The washing and lysis procedures conducted following incubation were both

Table 1: Amino Acid Sequences and Antimicrobial Activity of Bac 7 and Truncated Peptides[b]

Bac 7 peptides	Amino acid sequence	*E. coli*	*S. aureus*	*C. albicans*
1-59	RRIRPRPPRLPRPRPRP LPFPRPGPRPIPRP LPFPRPGPRPIPRP LPFPRPGPRPIPRP	0.24[a]	0.23	0.26
1-17	RRIRPRPPRLPRPRPRP	0.89	0.81	1.05
1-24	RRIRPRPPRLPRPRPRP LPFPRPG	0.45	2.6	1.12
26-59	PIPRP LPFPRPGPRPIPRP LPFPRPGPRPIPRP	1.16	1.38	1.44
30-59	P LPFPRPGPRPIPRP LPFPRPGPRPIPRP	5.84	30.5	0.5
44-59	P LPFPRPGPRPIPRP	>500	>500	30.5

[a] Minimal inhibitory concentration was measured in micromolar. [b] Repeat regions are underlined.

performed with PBS containing 0.5% sodium azide. For incubation at different temperatures, cells were added to 96-well plates and preincubated for 30 min at either 4 or 37 °C. After addition of peptide the plates were returned to the respective temperature for 30 min before washing and lysis.

Peptide−Protein Conjugation. Biotinylated peptides were synthesized on the solid phase using Boc chemistry. Following purification and confirmation of mass, the biotinylated peptides were stored at −20 °C. NeutrAvidin fluorescein conjugate (1 mg; Molecular Probes) was reconstituted in 500 μL of PBS and stored at −20 °C as a stock solution. The peptide−protein conjugates were used in the fluorescence quantitation assay according to the procedure of Pooga et al. (*28*). Briefly, biotinylated peptides and fluoresceinated protein were aliquoted into wells of a 96-well flat-bottomed plate at a 4:1 molar ratio (20 μM:5 μM peptide:protein). The total reaction volume was 50 μL. The reaction was left to proceed for 15 min before the addition of 150 μL of medium containing 3×10^5 murine monocytes. The quantitation of fluorescence was performed as described in the preceding section.

Confocal Microscopy. Cells at a concentration of 6×10^4 cells/mL were plated onto flat-bottomed 22 × 22 mm slide flask chambers (Nalgene Nunc International, Denmark) or P35G-0-14 dishes with a 14 mm diameter glass button chamber (MatTek Co., MA). The cells were incubated overnight at 37 °C. The fluoresceinated peptides were then added to the cell cultures and incubated for an additional hour. The cells were subsequently washed three times with PBS and fixed with 4% paraformaldehyde. The samples were covered with a no. 2 coverslip using mounting medium after air-drying. The fluorescence of cells was examined with a Zeiss LSM410 confocal microscope (Zeiss, Germany) equipped with a laser. Fluorescent images were obtained with a 488 nm band-pass filter for excitation. Software merging of images was carried out using COMOS software (Zeiss).

Cytotoxicity Assay. The cytotoxicity of the peptides was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Monolayer RAW 264.7 murine monocytes were transferred into a 96-well flat-bottomed plate (Corning, New York) at 1.5×10^5 cells/well (in 75 μL) and incubated for 16 h at 37 °C. Various concentrations of peptide solutions were added to the wells (in 25 μL), and the peptides and cells were incubated for either 1 or 24 h at 37 °C. Wells containing cells without peptides served as controls. Subsequently, 12.5 μL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for a further 4 h at 37 °C. During this 4 h incubation the mitochondrial succinate dehydrogenase of remnant viable cells converts MTT to formazan, which forms dark blue colored crystals in the wells. Following aspiration of excess MTT from the wells, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan to allow measurement of optical density (OD). The OD was measured at 590 nm using a Bio-Tek Instruments spectrophotometer. The cell viability is expressed as a percent ratio of A_{590} of cells treated with peptide over cells only.

CD Measurement. Peptides were dissolved in buffer solution containing 5 mM potassium phosphate, 5 mM sodium fluoride, and 0.02% sodium azide, with the pH adjusted to 7.0. Peptide concentration was determined using the Beer−Lambert law with absorbance measured at 257 nm (each peptide contains Phe) using a Shimadzu UVmini-1240 spectrophotometer. CD spectra were measured using a Jasco J-720 spectropolarimeter. Measurements were conducted with 0.2 nm resolution and a scan rate of 50 nm/min. Reported spectra are averages of 10 scans with smoothing by the J-700 system program (version 1.33.00). Spectra were measured at a peptide concentration of 100 μM and are corrected for buffer contribution.

RESULTS AND DISCUSSION

Analysis of Charged and Hydrophobic Regions of Bac 7. The 59-residue antimicrobial Bac 7 consists of a short cationic N-terminus and four copies of a 14-residue repeat (Figure 1). However, there is degeneration at the N-terminal repeat (N-repeat), which is significantly more basic than the three identical repeats at the C-terminus (C-repeat). The antimicrobial activities of the N-repeat and the C-repeats were assayed against a Gram-negative (*E. coli*) and a Gram-positive (*S. aureus*) bacterium as well as a fungus, *C. albicans*. As shown in Table 1, C-repeat alone did not exhibit any significant amount of antimicrobial activity. Longer peptides containing two or more of the C-repeats were necessary for producing antimicrobial activity. In contrast, N-repeat peptides, including the N-terminal 24-residue peptide (Bac_{1-24}), displayed high antimicrobial activity (Tables 1 and 2). The ability of Bac_{1-24} to traverse the cell membrane and localize to the cytoplasm was confirmed by confocal microscopy (Figure 2). Confocal microscopy is used to obtain "optical sections" through the cell, allowing the

Table 2: Amino Acid Sequences and Antimicrobial Activities of Truncated Bac_{1-24} Peptides[b]

Peptide	Amino acid sequence	*E. coli*	*S. aureus*	*C. albicans*
Bac_{1-24}	RRIR PRPPRLPRPRPRP LPFPRPG	0.45[a]	2.6	1.12
Bac_{1-17}	RRIR PRPPRLPRPRPRP	0.89	0.81	1.05
Bac_{1-15}	RRIR PRPPRLPRPRP	52.2	>500	28.8
Bac_{1-7}	RRIR PRP	>500	>500	>500
Bac_{5-24}	PRPPRLPRPRPRP LPFPRPG	18.1	>500	17.6
Bac_{7-24}	PPRLPRPRPRP LPFPRPG	39.8	>500	>500
Bac_{9-24}	RLPRPRPRP LPFPRPG	114	>500	>500
Bac_{11-24}	PRPRPRP LPFPRPG	>500	>500	>500
Bac_{13-24}	PRPRP LPFPRPG	>500	>500	>500
Bac_{15-24}	PRP LPFPRPG	>500	>500	>500

[a] Minimal inhibitory concentration was measured in micromolar. [b] Pro residues are underlined.

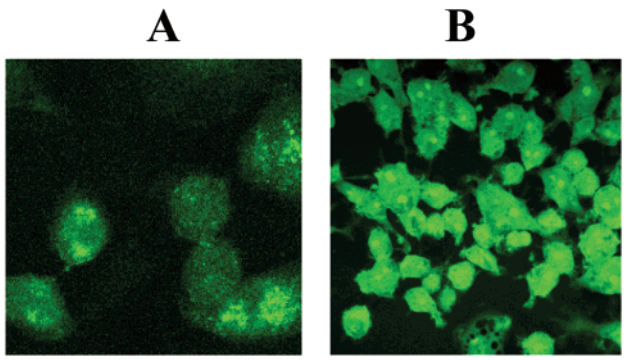

FIGURE 2: Confocal microscopy of murine monocyte cells after 1 h incubation with fluoresceinated peptides Bac_{1-24} (panel A) and the known cell-permeant peptide HIV Tat_{49-57} (RKKRRQRRR, panel B).

analysis of intracellular cross sections. In this case the technique has been used to show that the fluoresceinated peptides reside within the intracellular space and do not remain on the cell surface. The known cell-permeant peptide HIV Tat_{49-57} (RKKRRQRRR) localizes to the nucleus as shown by highly fluorescent regions within the cells (Figure 2B). Bac_{1-24} also enters the cells (Figure 2A); however, unlike Tat_{49-57}, it is dispersed throughout the intracellular space. These results suggest that the cell-permeant and antimicrobial activities of Bac 7 are colocalized at residues 1−24 of the N-terminus of Bac 7. Thus, our efforts to define the cell-permeant regions were focused on the peptide Bac_{1-24}.

Structural Dissection of Bac_{1-24} with Synthetic Peptides. The sequence of Bac_{1-24} is amphipathic and displays segregated charge and hydrophobic areas in three distinctive regions (Figure 3). The N-terminal charge-cap (N-cap) region is highly cationic. It contains a charge cluster of four residues, three of which are Arg residues. Immediately following the N-cap region is the PR-rich repeat region consisting of 12 amino acids that is rich in the Pro-Arg dipeptide. The PR repeat region is also charged, but the cationic residue Arg alternates with Pro. In contrast, the C-terminus of Bac_{1-24} contains the PX repeat region. It contains eight residues that are largely hydrophobic with a PX motif, with X being R, L, F, and G. Thus, the N-cap and the PR repeats represent

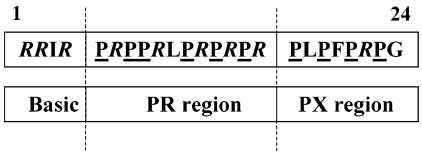

FIGURE 3: Structurally distinct regions of peptide Bac_{1-24}. The peptide is divided into three regions, separated by dotted lines, based on amino acid sequence. The dipeptide repeats that define the regions are listed below each segment.

the charged region whereas the PX repeat represents the hydrophobic region of Bac_{1-24}.

To determine the cell-permeant and microbicidal functions associated with each distinct region, Bac_{1-24} was systematically truncated from both termini using synthetic peptides (Table 2). Truncations from the C-terminus resulted in peptides Bac_{1-17}, Bac_{1-15}, and Bac_{1-7} that overlapped part or all of the charge-cap and PR-repeating regions and eliminated the hydrophobic PX repeat to produce increasing cationic peptides. In contrast, truncations from the N-terminus produced synthetic peptides Bac_{5-24}, Bac_{7-24}, Bac_{9-24}, Bac_{11-24}, Bac_{13-24}, and Bac_{15-24} with 20, 18, 16, 14, 12, and 10 residues, respectively, and incrementally eliminated the charge-cap and PR-repeating regions to afford peptides with increasing hydrophobicity. Despite their differences in length and cationic content, these truncated peptides are Pro-rich with the percentage of Pro remaining at approximately 50%.

Dissociation of Antimicrobial and Cell-Permeant Activities of Bac_{1-24}. The antimicrobial activity of each peptide was assayed against *E. coli*, *S. aureus*, and *C. albicans* (Table 2). Of all the peptides tested, only the parent peptide Bac_{1-24} and Bac_{1-17} were found to be active against all three microbes. The amphipathic nature of Bac_{1-24} with a cationic region appears to be important for antimicrobial activity. Deleting the C-terminal eight residues but maintaining the N-cap and the N-repeat sequence in Bac_{1-17} did not lead to loss of antimicrobial activity. However, further deletion such as Bac_{1-15} led to substantial loss of activity. Eliminating the cationic N-cap in peptides such as Bac_{5-24} resulted in low activity against *E. coli* and *C. albicans*. Similarly, peptides Bac_{7-24} and Bac_{9-24} with truncated PR repeats also displayed decreased activity against *E. coli* with the antimicrobial activity proportional to peptide length. The absence of the N-cap and six residues of the PR-rich region (peptide Bac_{11-24}) resulted in complete loss of antimicrobial activity. Bac_{13-24} and Bac_{15-24} did not display any measurable antimicrobial effect. Thus, by systematically truncating the N-terminus of Bac_{1-24} antimicrobial activity was eliminated.

The truncated peptides were coupled at their N-termini with a fluoresceinated carboxylic acid and tested by confocal microscopy to determine whether they possessed the cell-permeant activity, even though antimicrobial activity was absent. Unexpectedly, all tested peptides were cell-permeable, including short peptide fragments that did not conform to the known characteristics of cell-permeant peptides. For example, the cationic N-cap peptide of Bac_{1-7} (RRIRPRP) and PX-repeat peptide Bac_{15-24} (PRPLPFPRPG), containing four and two cationic charges, respectively, are neither highly cationic nor hydrophobic but rich in Pro, which may represent a new motif for cell-permeant peptides.

Cell-permeant activity was also quantified using an intracellular quantitation assay where cells were incubated in the

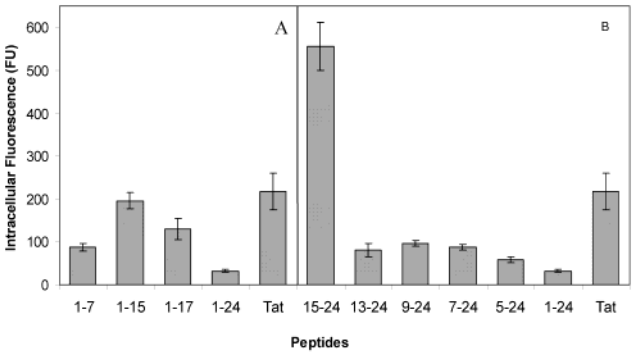

FIGURE 4: Comparison of cellular uptake of Bac 7 truncated peptides. Fluoresceinated peptides were incubated with murine monocytes at 5 μM for 30 min at 37 °C before washing and determination of intracellular fluorescence. The greatest uptake was observed in wells in which cells were exposed to Bac_{15-24}. The Tat_{49-57} peptide was included as a positive control. Peptides resulted from truncation of Bac_{1-24} from either the C-terminus (A) or N-terminus (B). Bac_{11-24} is not included here due to limited amount of the peptide but did fit the pattern in previous assays.

Table 3: Summary of Biological Activity of Truncated Bac_{1-24} Peptides

Peptide	Sequence	Activity	
		Antimicrobial[a]	Cell permeant[b]
Bac_{1-24}	RRIRPRPPRLPRPRPRPLPFPRPG	+++	+
Bac_{1-17}	RRIRPRPPRLPRPRPRP	+++	++
Bac_{1-15}	RRIRPRPPRLPRPRP	++	+++
Bac_{1-7}	RRIRPRP	-	++
Bac_{5-24}	PRPPRLPRPRPRPLPFPRPG	++	+
Bac_{7-24}	PPRLPRPRPRPLPFPRPG	+	++
Bac_{9-24}	RLPRPRPRPLPFPRPG	+	++
Bac_{11-24}	PRPRPRPLPFPRPG	-	++
Bac_{13-24}	PRPRPLPFPRPG	-	++
Bac_{15-24}	PRPLPFPRPG	-	+++

[a] Antimicrobial activity scale: +++, active against all strains tested; ++, active against some strains; +, weak activity; −, not active. [b] Cell-permeant activity scale: +++, ≥control; ++, <control; +, marginally greater than background.

presence of fluoresceinated peptides, and intracellular fluorescence was measured after removal of excess peptide. The level of intracellular fluorescence was compared to a positive control peptide, the highly charged cell-permeant peptide HIV Tat_{49-57} (Figure 4). All truncated peptides were found to be cell-permeant, and Bac_{1-15} and Bac_{15-24} exhibited activity similar to or better than the control Tat peptide. It is not surprising that Bac_{1-15} is cell-permeant because it shares characteristics with other cationic cell-permeant peptides with a high content of cationic residues (8 Arg) and is of similar length to the Tat peptide. However, the finding that short Bac peptides such as Bac_{1-7} and Bac_{15-24} are able to translocate across the cell membrane was unexpected.

Our results on the structure−activity study of Bac_{1-24} are summarized in Table 3. As shown in Figure 3, peptide Bac_{1-24} is divided into three regions, based initially on primary structure and dipeptide repeats. This arbitrary division may have functional significance in light of our results. For producing high and broad-spectrum antimicrobial activity, a cationic amphipathic primary structure consisting of the N-terminal two regions is required. In particular, the charged N-cap contains residues critical to the antimicrobial activity, as illustrated by Bac_{5-24}, whose activity is essentially abolished when the N-cap sequence is deleted. However, this short region alone is not capable of inducing an antimicrobial effect, even with three additional residues (Bac_{1-7}). The addition of the first complete N-repeat of the PR-rich region to the N-cap sequence as in Bac_{1-17}, but not the partial N-repeat as in Bac_{1-15}, restores high antimicrobial activity. The complete Bac 7 peptide consists of four copies of a 14-residue repeat (LPFPRPGPRPIPRP) with a 3-residue cationic sequence at the N-terminus. The first copy of this repeat, N-repeat, has degenerated and contains a high content of Arg residues in place of hydrophobic residues, creating the PR-rich region. On the basis of the current results, it is possible that the function of the PX-repeat region is to enable translocation of the peptide across the bacterial membrane after which the cationic PR region is available to interact with the protein synthesis machinery and affect bacterial cell killing. The need for a complete N-repeat may also be structural and will be discussed later under circular dichroism measurement.

Our working hypothesis is that cell-permeant peptides may use cationic antimicrobial peptides as templates because they contain half of their primary structural features. Our results generally substantiate this hypothesis and show that all truncated peptides of Bac_{1-24} are capable of translocating into cells. They include cationic peptides overlapping two regions, such as Bac_{1-7} and Bac_{1-15}, or hydrophobic peptides overlapping two repeats (N- and C-repeats), such as Bac_{13-24} and Bac_{15-24}. These peptides range in low (2) to high (9) Arg content, which does not appear to be a determining factor for translocation into cells. However, our results may have further extended the hypothesis, at least in the case of Pro-rich peptides, that the overlapping peptides of the cationic and hydrophobic regions, such as Bac_{9-24} and Bac_{11-24}, can also be useful as cell-permeant peptides.

Cell-Permeant Properties of Peptide Bac_{15-24}. Since Bac_{15-24}, a Pro-rich peptide, is neither highly cationic nor hydrophobic, unlike the known classes of cell-permeant peptides, this peptide was further characterized to determine whether it acted in a manner different to the other known cell-permeant peptides. To determine if uptake of Bac_{15-24} was energy dependent, the cell-permeant assay was performed in the presence of sodium azide to abolish energy-dependent uptake mechanisms (*29*). Incubation of cells with sodium azide did not inhibit the uptake of peptide Bac_{15-24} (data not shown), indicating that the mechanism utilized by Bac_{15-24} is not ATP-dependent. The effect of temperature on Bac_{15-24} translocation was also tested. Incubation at 4 °C significantly affected the uptake of Bac_{15-24}. Quantitative analysis of intracellular fluorescence showed that the intake of Bac_{15-24} was less than half of that recorded following incubation at 37 °C. The decrease in membrane fluidity at lower temperatures may have affected the uptake of this PR-rich peptide. Taken together, these results suggest that the mechanism of translocation of the Pro-rich peptide Bac_{15-24} is similar to the known cell-permeant peptides, such as the basic Tat peptide and the hydrophobic membrane-permeable sequences that are nonspecific and bidirectional.

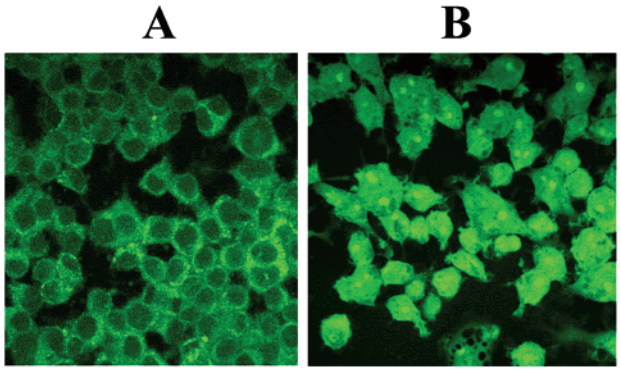

FIGURE 5: Confocal microscopy of murine monocytes after 1 h incubation with fluoresceinated cell-permeant peptides. Cells were incubated with two different peptides, Bac_{15-24} (A) and Tat_{49-57} (B). The peptide Tat_{49-57} translocates into the nucleus whereas Bac_{15-24} remains in the cytoplasm.

Approximately 1% of the total Bac_{15-24} was found within the cell using the quantitation assay. The amount of peptide that translocated into the intracellular environment was calculated by subtracting the amount of peptide remaining in the medium after incubation from the starting material. In a similar experiment performed by Lindgren et al. (*30*), transportan and penetratin were labeled with anthranilic acid, and 6.7% and 3.3% of transportan and penetratin were internalized after 30 min, respectively. Because the translocation of peptide is bidirectional, the 1% uptake may not accurately represent the true amount of peptide that translocates into the cells. This bidirectional nature of cell-permeant peptides may be advantageous for therapeutic use where peptides must migrate through different cell types to reach the target tissue.

Localization of Cell-Permeant Peptides. Confocal microscopy was employed to determine intracellular peptide location. Cells were incubated in the presence of either fluoresceinated Bac peptides or the positive control peptide Tat_{49-57} for 1 h before fixing and observation by confocal microscopy (Figure 5). In general, our results showed that the highly cationic Tat_{49-57} peptide and Bac peptides, containing the N-cap and the PR-repeat region such as fluoresceinated Bac_{1-15}, Bac_{1-17}, and Bac_{1-24}, were localized in both the cytoplasm and nucleus (unpublished experiments). These peptides contain a cluster of seven or more cationic charges. In contrast, peptides with four or less cationic charges, including the moderate and lowly cationic charged Bac peptides without the N-cap and most of the PR-region such as Bac_{15-24}, were found to localize mainly within the cytoplasm. From the confocal microscopy images, we can also conclude that Bac_{15-24} resides within the cytoplasm and does not remain attached to, or embedded within, the cell membrane. Our results are consistent with the previous findings by others who show that highly cationic peptides such as Tat_{49-57} enter both the cytoplasm and the nucleus. Even though the mechanism used by cationic peptides to enter the nucleus has not been confirmed, the ability of Tat_{49-57} to penetrate the nucleus is likely due to the interaction of the cationic residues with the importin transport complex (*31*, *32*). This importin complex is utilized by cytoplasmic proteins for nuclear entry through interaction of importin proteins with their cationic nuclear localization sequence (NLS; *33*−*35*). In contrast, lowly cationic charged peptides such as Bac_{15-24} and the hydrophobic peptides from the signal sequences are unable to use the same mechanism for nuclear entry and remain within the cytoplasm.

Table 4: Cytotoxicity of Bac_{15-24} and Tat_{49-57}

peptide	concn (μM)	% cell viability 1 h[a]	% cell viability 24 h
Bac_{15-24}	0.5[b]	92 ± 5	98 ± 7
	20	87 ± 21	74 ± 11
Tat_{49-57}	0.5	85 ± 7	88 ± 24
	20	89 ± 18	91 ± 21

[a] Peptide incubation time. [b] Peptide concentration.

Cytotoxicity of PR-Rich Peptide Bac_{15-24}. Previously, we have shown that Bac_{1-24} is nontoxic to mammalian cells (unpublished experiments). An MTT assay was employed to determine if the PR-rich peptide Bac_{15-24} was cytotoxic. Cells were incubated for 1 or 24 h in the presence of either Bac_{15-24} or Tat_{49-57}, and cell viability was measured by the addition of MTT, which is converted by live cells to formazan, resulting in a color change that was monitored spectrophotometrically. After cells were exposed to peptides for 1 h, at least 85% remained viable, whereas >74% of cells remained viable after incubation with peptides for 24 h (Table 4). Our results conclude that the PR-rich Bac_{15-24} is no more toxic than the Tat_{49-57} control peptide, even after incubation at the relatively high concentration of 20 μM for 24 h.

Interestingly, a lack of cytotoxicity was also observed in cells incubated with the larger Bac_{1-24} peptide. This indicates that even though this peptide does indeed possess antimicrobial activity (Table 2), it is not toxic toward mammalian cells. Thus, the low intracellular fluorescence determined in the quantitation assay cannot be attributed to the peptide inducing cell lysis and, thereby, releasing peptide associated with the cells into the medium to be washed out during the assay.

Circular Dichroism Analysis. Circular dichroism (CD) analysis was performed to determine the conformation of Bac_{15-24}. Because 50% of the residues in Bac_{15-24} are the α-helix-breaking proline, Pro-rich peptides do not form α-helices, but rather they adopt a left-handed polyproline type II (PPII) structure. Because the PPII structure is the conformation preferred by homopolymers of proline, we prepared a proline homopolymer $Pro_{10}Gly$ ($P_{10}G$) for comparison (*36*). Figure 6 shows the CD spectrum of peptide Bac_{15-24} and $P_{10}G$. Also included in this figure are the spectra of Bac_{13-24}, Bac_{11-24}, and Bac_{9-24} to highlight the features of the Bac_{15-24} spectrum. The $P_{10}G$ peptide possesses a CD spectrum characteristic of a PPII helical conformation with positive and negative bands at 228 and 205 nm, respectively. Each Pro-rich peptide, with the exception of Bac_{15-24}, displays double minima around 204 nm that decrease in intensity with the decrease in peptide length. The Bac_{15-24} spectrum also deviates from the other Pro-rich peptides in that there is a slight upward rise at 229 nm, indicative of the PPII helix. Our CD measurements suggest that the Pro-rich peptides contain an ordered state and are a hybrid of the spectra typically generated by peptides with either high α-helical content or PPII content. The minima displayed around 205 nm infer polyproline type II helices, whereas the doublet is usually found in spectra of α-helical peptides.

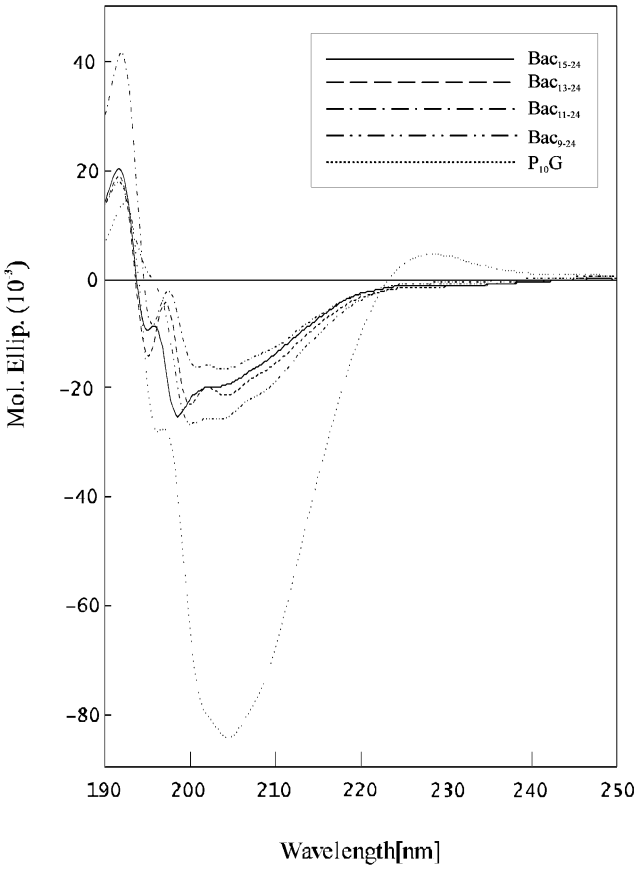

FIGURE 6: Circular dichroism spectra for Pro-rich peptides in 100 mM aqueous buffer.

The PPII helix forms an extended structure and consists of three residues per turn, compared to 3.4 of the α-helix, and thus the hybrid may have between 3 and 3.4 residues per turn. This hybrid conformation may also possess a constrained conformation due to the rigidity enforced by proline on surrounding amino acids with ϕ and ψ angles of −78° and +146°, respectively. This observation is in agreement with several other studies (*14*, *37*−*39*). Tani et al. (*14*) measured the CD spectra of two other Bac 7 peptides, RRIRPRPPRLPRPRPRP and LPFPRPGPRPIPRP, and concluded that these peptides both take unique conformations that are different from the regular α-helix, β-structure, PPII helix, and the unordered state. According to Raj et al. (*37*) Pro-rich Bac 5 and its synthetic fragments also retain a single-stranded extended helical structure in both aqueous and nonaqueous solvents of varying polarity. Similarly, others have shown, using both CD analysis and energy minimization modeling techniques, that Pro-rich peptides possess stable conformations that closely resemble the PPII conformation (*38*, *39*).

Delivery of Protein Cargo by Pro-Rich Peptides. The ability of Bac_{1-24} and Bac_{15-24} to act as transporting vectors to import a protein cargo into cells was performed using a method described by Pooga et al. (*28*). In this method, the Bac_{15-24} and Bac_{1-24} peptides were attached noncovalently through the biotin−avidin complex to the cargo, the fluoresceinated protein NeutrAvidin. The noncovalent attachment of both translocating peptides to the protein provides a convenient method to determine the usefulness of the cell-permeant peptides. The amount of protein transported into cells was then determined by the quantitative assay previously described. The peptides were biotinylated at their N-termini and mixed separately at a 4:1 molar ratio with the fluoresceinated 60 kDa NeutrAvidin protein prior to addition of cells. The results (Figure 7) showed that the PR-rich peptides increased the quantity of internalized protein at least 4-fold when compared to the background using NeutrAvidin alone. Bac_{1-24} was a better vector than Bac_{15-24}. The size of the peptide vectors may have a significant influence on this result. The peptide binding site of avidin−NeutrAvidin is relatively deep, approximately four residues. Thus, the number of exposed residues of the 10-residue Bac_{15-24}, after forming the biotin−avidin complex, is effectively halved, resulting in a short 6-residue sequence projecting from the protein surface to affect its translocating function. In contrast, the 24-residue Bac_{1-24} would have sufficient exposed length after forming the complex. A disadvantage associated with the use of the 24-residue peptide, however, is that the peptide−protein conjugate is fairly insoluble. This insolubility limits the concentration of the conjugate in solution and therefore increases the amount of material required for effective translocation of the protein cargo.

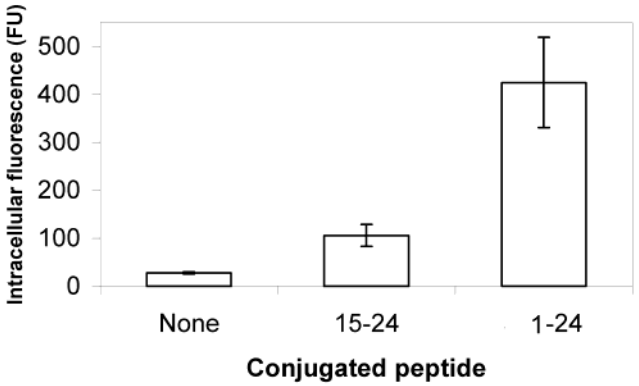

FIGURE 7: Ability of Pro-rich peptides to deliver protein cargo. Biotinylated peptides were mixed in wells with NeutrAvidin fluorescein conjugate for 15 min prior to addition of cells. Following 30 min incubation the cells were washed and lysed, and the soluble fraction was tested for fluorescence.

CONCLUSIONS

The PR-rich antimicrobial peptide Bac 7 kills bacteria in a two-stage process (*14*, *15*). It first gains entry to cytoplasm and then inhibits intracellular targets. This killing mechanism is different from the membranolytic mechanism frequently used by pore-forming antimicrobial peptides such as cecropins, magainins, tachyplesin, and defensins. Several other PR-rich antimicrobial peptides may act in a similar fashion. Apidaecin has been reported to act by traversing the bacterial inner and outer membranes and then specifically inhibiting protein synthesis in a dose-dependent manner (*25*, *40*). PR-39, an antimicrobial PR-rich member of the cathelicidin family, acts by halting protein and DNA synthesis (*20*). Our work aimed to separate the translocating from the bacteriocidal activity of Bac 7 and to exploit the Bac 7 template to design novel cell-permeant peptides. In this regard, we have successfully dissociated the cell-permeant activity of Bac 7 from the antimicrobial activity and showed that the translocating regions are capable of acting as a vector for

delivering large cargo. Furthermore, we have shown that there are multiple translocating regions and that most contain a novel repeating $(PX)_n$ motif that is different from all other known cell-permeant peptides. The $(PX)_n$ motif possesses both hydrophobic character and a distinct secondary structure, both of which may be implicated in the translocation activity. Proline is a hydrophobic imino acid and, along with the other hydrophobic residues that are found in the $(PX)_n$ motif of Bac 7, creates a peptide capable of interacting with the lipid tails that constitute the cell membrane. The high content of proline in the Bac 7 peptides confers on these sequences a conformation containing a hybrid of the PPII helix and the α-helix. In addition, our results show that Bac 7 equips its sequence with multiple cell-permeant regions capable of translocating to both cytoplasm and nucleus, suggesting that Bac 7 or its proteolytic degraded fragments possess multiple intracellular targets as killing mechanisms.

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