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Antibacterial potential of hGlyrichin encoded by a human gene

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Emerging multidrug-resistant (MDR) bacteria are an enormous threat to human life because of their resistance to currently available antibiotics. The genes encoding antibacterial peptides have been studied extensively and are excellent candidates for a new generation of antibiotic drugs to fight MDR bacteria. In contrast to traditional antibiotics, antibacterial peptides, which do not cause drug resistance, have an unparalleled advantage. However, because most antibacterial peptides originate in species other than humans, the hetero-immunological rejection of antibacterial peptides is a key disadvantage that limits their clinical application. In this study, we identify hGlyrichin as a potential human antibacterial polypeptide. The hGlyrichin polypeptide kills a variety of bacteria including the MDR bacteria methicillin-resistant *Staphylococcus aureus*, MDR *Pseudomonas aeruginosa*, and MDR tubercle bacillus. A 19 amino acid peptide (pCM19) at positions 42–60 of hGlyrichin is crucial for its antibacterial activity. The hGlyrichin polypeptide kills bacteria through the destruction of the bacterial membrane. In addition, all peptides that are homologous to hGlyrichin have antibacterial activity and can penetrate the bacterial membrane. Importantly, hGlyrichin does not cause hemolytic side effects *in vitro* or *in vivo*. Therefore, based on the virtues of hGlyrichin, i.e., the absence of hetero-immunological rejection and hemolytic side effects and the unambiguous efficacy of killing pathogenic MDR bacteria, we propose hGlyrichin as a potential human antibacterial polypeptide. **Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.**

Supporting information may be found in the online version of this article.

Keywords: hGlyrichin; antibacterial peptides; multidrug-resistant (MDR) bacteria; human gene

Introduction

When examining hematopoietic regulatory factors using subtractive hybridization methods in two kinds of mouse bone marrow stromal cells [long-term culture (LTC) and non-LTC], we identified 131 differentially expressed sequence tag (EST) clones. Among these EST clones, seven were without functional information and were selected for further investigation. When these seven cDNA clones were expressed in *Escherichia coli* BL-21, surprisingly, the inducible expression of one, AY278457, resulted in a bacterial growth delay. Based on these results, we speculated that this gene might encode an antibacterial peptide that can kill bacteria. Because this gene product, a 79 amino acid polypeptide, consists of 21% glycine residues, it was designated hGlyrichin (the abbreviation of *h*uman *gly*cine *rich* prote*in*).

A database search showed that hGlyrichin is an evolutionarily highly conserved gene. The human form of hGlyrichin is 100% homologous to the form of the gene found in all mammalian species analyzed. During our investigation of the antibacterial properties of hGlyrichin, Chung *et al*. 2006 named this same gene Romo1 (reactive oxygen species modulator 1) and showed that it encodes a protein that is identical to hGlyrichin. They reported that Romo1 (hGlyrichin) is an integral mitochondrial inner-membrane protein that induces an increase in reactive oxygen species (ROS) that is proportional to its expression level [1]. Romo1 plays an important role in redox signaling during proliferation in normal cells [2] and cancer cells [3]. The increased expression of Romo1 is associated with an increased level of ROS, which results in senescence in aged cells [4] and chemotherapy resistance in cancer cells [5]. Meanwhile, Zhao *et al*. 2009 [6] named this same gene product mitochondrial targeting GxxxG motif (MTGM) because of the presence of a GxxxG motif in its transmembrane region and indicated that MTGM (hGlyrichin) coordinately regulates mitochondrial morphology and cell proliferation.

In this study, we show that hGlyrichin possesses antibacterial activity. We report that the exogenous expression of hGlyrichin results in a growth delay of *E. coli* BL-21 through destruction of the bacterial membrane. The pCM19 peptide of hGlyrichin, corresponding to amino acids 42 to 60, is crucial for exerting

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Abbreviations: MDR, multidrug-resistant; MRSA, *Staphylococcus aureus*; MDR-PA, MDR *Pseudomonas aeruginosa*; MDR-TB, MDR tubercle bacillus; ROS, reactive oxygen species; LB, Luria–Bertani; MH, Mueller–Hinton; IPTG, isopropyl β-D-1-thiogalactopyranoside; MIC, minimal inhibitory concentration; PBS, phosphate-buffered saline; FACS, fluorescence activated cell sorter; GFP, green florescence protein; EST, expressed sequence tag; CDS, cDNA coding sequence; PI, propidium iodide; AMP, antimicrobial peptide; ECL, enhanced chemiluminescence.

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the antibacterial activity of hGlyrichin. The pCM19 peptide not only kills *E. coli* BL-21, but it also kills a variety of other athogenic bacteria. Incubating pCM19 with blood or the intravenous injection of pCM19 into mice does not result in hemolysis, suggesting that hGlyrichin is potentially an antibacterial peptide that does not cause side effects. Therefore, we propose that hGlyrichin is an effective substitute for traditional antibiotics to cure severe infectious diseases that are caused by newly emerging multidrug-resistant (MDR) bacteria

Materials and Methods

Ethics Statement

Mice experiments were conducted at the animal facility of Beijing Institute of Radiation Medicine. General animal care was provided under an Animal Use Protocol approved by the Institutional Animal Care and Use Committee at the Beijing Institute of Radiation Medicine. (Approval ID: 07/M038).

Human blood was collected from healthy volunteers who gave written informed consent for venipuncture, as approved by the Institutional Review Board of the Beijing 307 Hospital.

Reagents and Antibodies

Restriction endonucleases, T4 DNA ligase, Taq DNA polymerase, and DNA Marker DL-2000 were from Takara (Dalian, China). The plasmid extraction kit, DNA fragment purification and recovery kit, RNA purification kit, and random primer gene labeling system were from Promega (Madison, WI, USA). The enhanced chemiluminescence (ECL) luminescence kit was from Thermo Fisher Scientific Inc. (Rockford, IL, USA). The RPMI-1640 culture medium, fetal bovine serum, and trypsin were from Invitrogen (Carlsbad, CA, USA). The yeast extract and peptone were from Oxiod Ltd. (Hampshire, UK). The primer synthesis and sequencing were carried out by Invitrogen Bioengineering (Shanghai, China). The green florescence protein (GFP) antibody was from Calbiochem (San Diego, CA, USA). The secondary antibodies were from eBioscience (San Diego, CA, USA).

Plasmids

The plasmid pTAT-EGFP was constructed as previously reported [7]. The plasmid pGEX4T-2-hGlyrichin was constructed using the following primers: sense, *GAA TTC* CC ATG CCG GTG GCC GTG GGT CC; anti-sense, *CTC GAG* T GCA TCG GAT GCC CAT CCC AA. The plasmid pGEX4T-2-pTH was constructed using the following primers: sense, *GAA TTC* CC ATG ATA CCT GCA AAA GAC AT; anti-sense, *CTC GAG* T CTG GGA TTT AGC TTT AGT TA.

Bacteria Strains, Culture, and Treatment

Escherichia coli, *Salmonella typhi*, *Staphylococcus aureus*, ampicillin-resistant *S. aureus*, *Neisseria meningitidis*, *Yersinia pestis*, methicillin-resistant *S. aureus* (MRSA), multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA), and multidrug-resistant tubercle bacillus (MDR-TB) were kept at the Microbial Testing Center of the Academy of Military Medical Science (20 Dongdajie Road, Beijing). Bacteria were maintained in Luria–Bertani (LB) or Mueller–Hinton (MH) liquid media or on solid agar plates. All treatments were carried out by incubating the bacterial cells with various peptides. In each experiment, bacteria treated with normal saline were included as the negative controls, and bacteria treated with ampicillin were included as the traditional antibiotic control. Following incubation, bacteria were cultured for different times and were used for further experiments.

Bioinformatics Software

TMHMM software was used for predicting transmembrane regions, CLUSTALW software was used for aligning multiple sequences, SignalP3.0 and TargetP 1.1 were used for predicting signal peptide sequences, and PHYLIP software was used to evaluate evolutionary distance.

Bacterial Growth Curve Determination

A bacterial growth curve was generated following the transformation of *E. coli* with pGEX4T-2 (empty vector, negative control), pGEX4T-2-pTH (non-antimicrobial activity peptide, mock control) or pGEX4T-2-hGlyrichin. The transformed *E. coli* were treated or not treated with 0.50 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the growth was determined by measuring the OD600 on a plate reader after incubation for different periods as indicated in each figure. The growth curve was expressed as $Log10^{OD600}$ versus time.

Peptide Synthesis

All peptides were synthesized by GL Biochem (Shanghai, China), and their sequences are as follows: pA, MPVAVGPYGQSQPSCFDRVKMGFVMGCAVG; pB, MAAGALFGTFSCLRIG; pD, MQSGG TFGTFMAIGMGIRC; pCM19 (human), CLRIGMRGRELMGGIGKTM; pGE19 (human, a random composition of pCM19), GICRLMMRRGLETIGGKMG; pGT18, (*Drosophila melanogaster*), ALRYGLRGRELINNVGKT; pAT18 (*Arabidopsis thaliana*), AIRVKVPGLHKVRFIGQTT; pIM18, (*Schizosaccharomyces pombe*), AIATQGPGPDGVVRTLGK; SC-22 (human, fragments of BPI-P2, positive control), SKISGKWKAQKRFLKMSGNFGC.

Determining the MIC of pCM19 Using the Agar Plate Dilution Method

The pCM19 peptide was dissolved in water and added to molten MH agar. Dilutions of pCM19 were prepared in sterile distilled water at a 10× concentration. The minimal inhibitory concentration (MIC) range was 5, 10, 20, 40, 80, 160, 320, 640, and 1280 μg/ml of pCM19. The estimation of the MIC followed established protocols [8,9].

Determining the Antimicrobial Activity of pCM19 Using the Agar Plate Method

The antimicrobial activity of pCM19 (1000 μg/ml) was measured using the agar plate method. The preparation of pCM19, MH agar plates, and inoculums for the MIC test and inoculation and incubation of the medium followed established protocols [8,9].

Detection of Propidium Iodide Influx by FACS

The bacteria were cultured in liquid LB media at 37 °C to an $OD_{600} = 0.4$ and were co-cultured with the various peptides, normal saline (negative control), or ampicillin (traditional antibiotic control) for different times as indicated in each figure. Bacteria were collected by centrifugation at 1500 g and were fixed with ice-cold 70% (*v/v*) ethanol. The pellets were resuspended in

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phosphate-buffered saline (PBS) containing propidium iodide (PI) (50 μg/ml) and DNase-free RNase (1 μg/ml) and were incubated for 1 h before analysis using a fluorescence activated cell sorter (FACScan) flow cytometer.

Scanning Electronic Microscopy

Escherichia coli cells were treated with 1 mg/ml pCM19, the negative control peptide, or normal saline and were simultaneously fixed and permeabilized using 2% gluteraldehyde and 0.5% Triton X-100 in PBS. The cells were embedded and sectioned parallel with the substratum using a Reichert Jung Ultracut E/S microtome. The serial thin sections (60–70 nm thick) of the bacterial cells were stained with uranium acetate for 10 min, washed three times with Tris-buffered saline, stained with lead citrate for 10 min, and photographed using a JEOL JSM-S600LV electron microscope (JEOL Inc. Peabody, MA, USA).

GFP Expression and GFP Release

The vector pTAT-EGFP [7,10] was transformed into *E. coli* to allow the expression of a soluble fusion protein containing GFP and the HIV-1 TAT protein. TAT-EGFP protein expression was detected in samples of transformed *E. coli* by western blotting using a GFP antibody. The release of pTAT-EGFP from the bacteria into the culture medium was detected in samples of culture medium by western blotting using a GFP antibody.

Western Blotting

The samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were blotted onto a nitrocellulose membrane. The membrane was incubated with a specific primary antibody overnight at 4 °C followed by incubation with the horseradish peroxidase-conjugated secondary antibody for 1 h. Protein bands were visualized using the ECL western blotting detection system (Amersham, Arlington Heights, IL, USA).

In Vitro Hemolysis Assay

Blood samples from healthy donors were treated with heparin to avoid clotting. The blood was washed three times with normal saline, and 8 μl samples were diluted to 100 μl in normal saline. The diluted blood (100 μl) was incubated with 100 μl of 100, 200, 500, 1000, 2000, or 3000 μg/ml pCM19 for 1 h at 37 °C. Centrifugation was at 1000 *g* for 5 min at 4 °C. Each treatment was performed in triplicate. The absorbance of each sample was measured at a wavelength of 570 nm using microplate reader. The control group contained samples treated with 100 μl normal saline (negative control), 0.1% TritonX-100 (positive control), or 4800 μM ampicillin (traditional antibiotic), respectively, and controls were carried out in triplicate

In Vivo Hemolysis Assay

The hemolysis assay was conducted in 8-week-old adult male Balb/c mice (body weight: 20–25 g). The mice were divided into three groups of five mice. The first (control) group was injected with 200 μl 0.9% NaCl; the second and the third groups were injected with 200 μl pCM-19. In the second group, the amount of pCM19 injected was 5 mg of pCM19 per kilogram of body weight. In the third group, the amount of pCM19 injected was 10 mg of pCM19 per kilogram of body weight. At 2 or 5 h after treatment, blood samples (100 μl) were collected by intravenous hemospasia. The blood was analyzed using an automatic blood cell analyzer (Sysmex F-820). The numbers of white and red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelets were counted.

Statistical Analysis

The data are presented as the means ± SD. Statistical significance among groups was determined using a paired, two-tailed Student's *t*-test with SAS software (version 9). The findings were considered significant at $p < 0.05$.

Results

Expression of hGlyrichin in *E. coli* BL-21 (DE3+) Leads to Bacterial Growth Delay

Unless otherwise stated, the BL-21 (DE3+) strain of *E. coli* was used for these experiments. To produce the exogenously expressed hGlyrichin protein for functional studies, we inserted the fragment containing the hGlyrichin cDNA coding sequence into the prokaryotic expression vector pGEX4T-2 to produce the plasmid of pGEX4T-2-hGlyrichin. Meanwhile, we constructed the plasmid pGEX4T-2-pTH (GI: 238690128) to be used as a non-relevant mock control for vector transformation and exogenous protein expression [11,12]. Because pTH is a human gene known to have no antibacterial activity, it was an appropriate choice for use in this context. *E. coli* transformed with pGEX4T-2 (empty vector, negative control), pGEX4T-2-hGlyrichin, or pGEX4T-2-pTH were cultured in LB media in the presence or absence of IPTG. A bacterial growth curve indicated that the inducible expression of hGlyrichin but not of pGEX4T-2 or pGEX4T-2-pTH resulted in a significant bacterial growth delay at the exponential (5–11 h) and stationary (12–20 h) phases (Figure 1A). The delayed growth was not because of the transformation of an exogenous gene because the *E. coli* containing pTH did not exhibit a growth delay in the presence of IPTG (Figure 1A), demonstrating that hGlyrichin has antibacterial activity.

The Amino Acids (pCM19) at Positions 42 to 60 are Crucial Residues for the Antibacterial Activity of the hGlyrichin Peptide

To identify the critical amino acid residues within hGlyrichin that are responsible for its antibacterial activity, we used bioinformatics software to analyze the sequence of hGlyrichin. TMHMM analysis indicated that the transmembrane domain is located at amino acid positions 21–44, and the inner-membrane domain is located at residues 45–79. Analysis using SignalP3.0 software showed that the leading peptide (signal peptide) is located at residues 1–20. Therefore, we synthesized four peptides: pA (1–30 AA), pB (31–46 AA), pCM19 (42–60 AA) and pD (61–79 AA) (Figure 1B) to represent these three domains.

When we incubated the four peptides with *E. coli*, only pCM19 stalled bacterial growth. Furthermore, we found that at a concentration of 50 μg/ml, the antibacterial activity of pCM19 against *E. coli* was 100% (Figure 1C).

Plate cultures of bacteria with 1000 μg/ml pCM19 on the agar surface showed that pCM19 killed the eight bacterial strains tested including *S. typhi*, *S. aureus*, ampicillin-resistant *S. aureus*,

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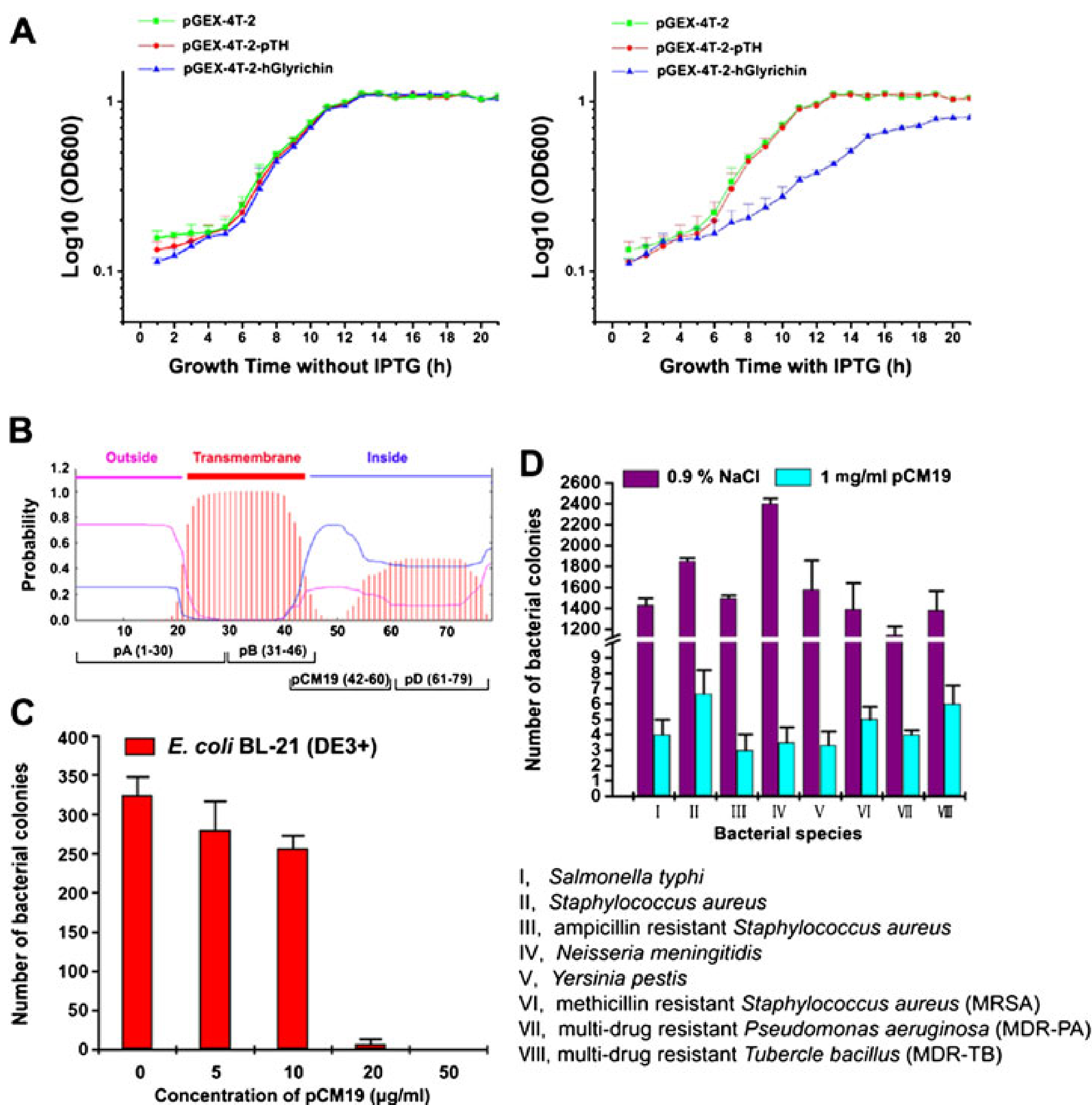

Figure 1. Confirming antibacterial activity and effective key residues of hGlyrichin. (A) The bacterial growth curve of *E. coli* transformed with pGEX4T-2 (empty vector, negative control), pGEX4T-2-pTH (non-antimicrobial activity peptide control), or pGEX4T-2-hGlyrichin in the absence (left panel) or presence (right panel) of 0.5 mM IPTG. The OD600 value was measured, and the growth curve was expressed as Log10OD600. Data are expressed as the mean ± SD (*N* = 3). (B) TMHMM was used to predict the transmembrane region of hGlyrichin. The peptides pA, pB, pCM19, and pD were synthesized. (C) Dose effect of pCM19 killing of *E. coli*. (D) pCM19 can kill a variety of pathogenic bacteria. Bacteria were cultured on MH agar plate with or without 1000 μg/ml pCM19. Data are expressed as the mean ± SD (*N* = 3).

N. meningitidis, *Y. pestis*, MRSA, MDR-PA, and MDR-TB (Figure 1D). Among these eight bacterial strains, MRSA, MDR-PA, and MDR-TB are MDR bacteria. The MIC of pCM19 against these pathogenic bacteria is approximately 300 μg/ml. These results show that the crucial residues for the antibacterial activity of hGlyrichin are at amino acid positions 42–60, demonstrating that pCM19 can represent hGlyrichin for functional investigations, and indicate that hGlyrichin has the potential to kill pathogenic MDR bacteria.

The pCM19 Peptide of hGlyrichin Kills Bacteria by Inducing Bacterial Membrane Breakage

Traditional antibiotics kill bacteria primarily by inhibiting the synthesis of bacterial DNA or proteins, and this is largely responsible for the induction of the MDR phenotype. Antibacterial peptides kill bacteria by destroying the bacterial membrane and do not affect the synthesis of bacterial DNA or proteins, and this is the main reason why they do not induce the MDR phenotype [13].

We next examined if hGlyrichin uses the same mechanism as other antibacterial peptides to kill bacteria, namely by destroying the bacterial membrane. We selected PI as a staining dye to distinguish whether or not the bacterial membrane was broken after incubation with pCM19. PI can enter a cell with a broken membrane but cannot enter one with an intact membrane. DNA flow cytometry analysis showed that approximately 50% of the *E. coli* cells were stained with PI after 10, 20, or 30 min incubation with 1000 μg/ml pCM19 but not with normal saline (0.9% NaCl solution) or in the absence of any detergent (Figure 2A

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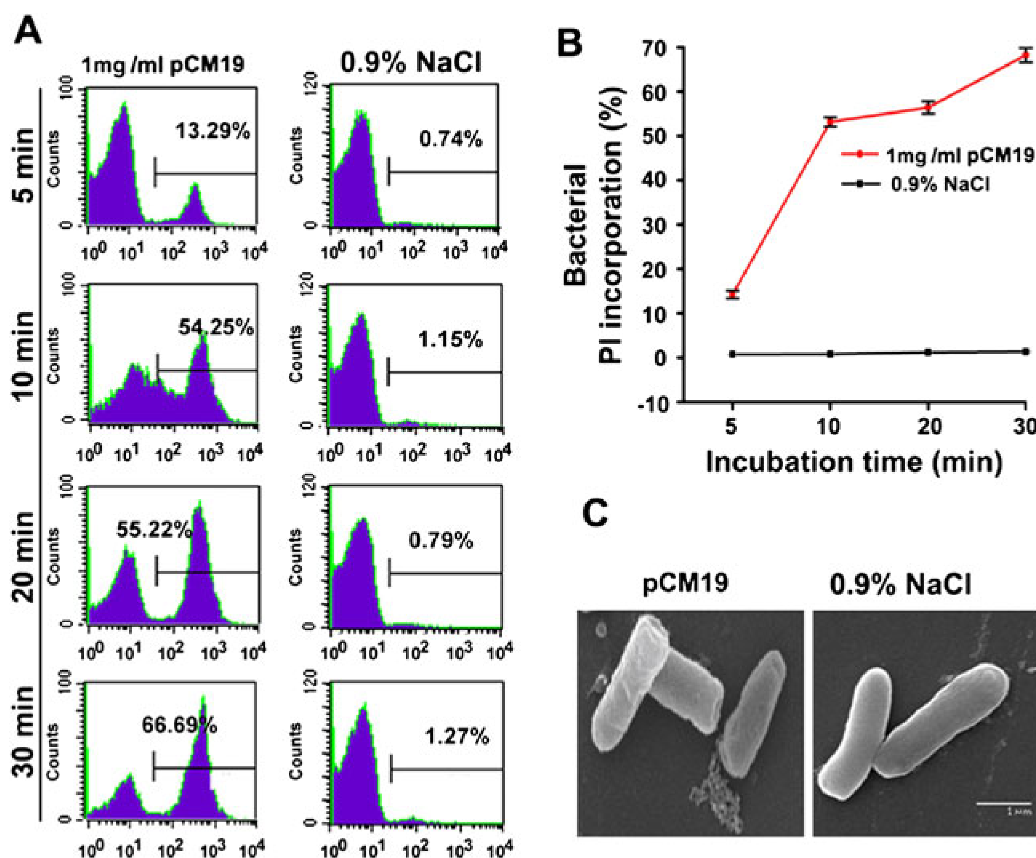

Figure 2. pCM19 peptide of hGlyrichin kill bacteria by destroying the bacterial membrane. (A) Representative results of flow cytometric analysis of membrane breakage in *E. coli* through PI incorporation 5, 10, 20, and 30 min post-incubation with 1000 μg/ml pCM19 in the absence of detergent. Incubation with 0.9% NaCl served as negative control. (B) Flow cytometric analysis as indicated in A. Data are expressed as the mean ± SD ($N = 3$). (C). Electron microscopy of *E. coli* incubated with pCM19. Left panel, incubation with 1000 μg/ml pCM19; right panel, incubation with 0.9% NaCl (negative control) (bar = 1 μm).

and B). The results of electron microscopy confirmed these observations. Incubation of *E. coli* with 1000 μg/ml pCM19 resulted in the leakage of cytoplasmic components through the membrane, whereas incubation with 0.9% NaCl did not (Figure 2C). The above data clearly demonstrate that hGlyrichin kills bacteria by destroying the bacterial membrane in a manner similar to other well-known antibacterial peptides, and this implies that hGlyrichin is an excellent candidate for use as a protein antibiotic that does not induce the antibiotic-resistance phenotype in bacteria.

Proteins Homologous to hGlyrichin

Proteins that are homologous to hGlyrichin were identified using BlastP software to search databases. Homologous proteins from divergent species with scores of above 40 that exhibited similar characteristics to hGlyrichin were listed (Tables S1 and S2). The evolutionary trees were generated using Phylip software (Figure S1). Based on evolutionary distances (Figure S1), the sequences homologous to hGlyrichin were divided into four groups and were analyzed with a multiple sequence alignment tool using ClustalX software (Figure S2). The homology of sequences to hGlyrichin in the first group was approximately 100%. Because pCM19 can represent hGlyrichin for its antibacterial activity, we used the synthesized (human) pCM19 peptide to represent all of the homologous proteins in the first group. The pGT18 peptide from *Caenorhabditis elegans*, the pAT18 peptide from *A. thaliana*, and the pIM18 peptide from *S. pombe* were synthesized to represent the members of the second, third, and fourth groups, respectively (Figure S2).

The Peptides Homologous to pCM19 Exhibited Antibacterial Potency and Were Capable of Penetrating Bacterial Membranes

It has been reported that many of the known antibacterial peptides, such as BPI-P2 [14] and temporin, can disrupt the microbial membrane [15–17]. Therefore, to represent well-known antibacterial peptides, we synthesized the SC-22 peptide fragment of BPI-P2 as a positive control for our experiments [18,19]. The pGE19 peptide, which is randomly composed of residues from pCM19, was used as a negative control for pCM19. Normal saline and ampicillin were used as a negative control and a traditional antibiotic control, respectively (Figure 3A).

DNA flow cytometry analysis showed that in the absence of detergent the proportion of *E. coli* stained with PI was approximately 55%, 44%, 42%, 25%, or 65% after 20 min incubation with pCM19, pGT18, pAT18, pIM18, or SC-22, respectively. In contrast, *E. coli* was not stained with PI after 20 min incubation with 1000 μg/ml pGE19, normal saline, or ampicillin. The data demonstrate that all the hGlyrichin homologous proteins tested are antibacterial peptides and that the human ortholog hGlyrichin is the most efficient at killing bacteria.

The vector pTAT-EGFP has been widely used to produce the soluble GFP-HIV-1-TAT fusion protein in *E. coli* [7,10]. The pTAT-

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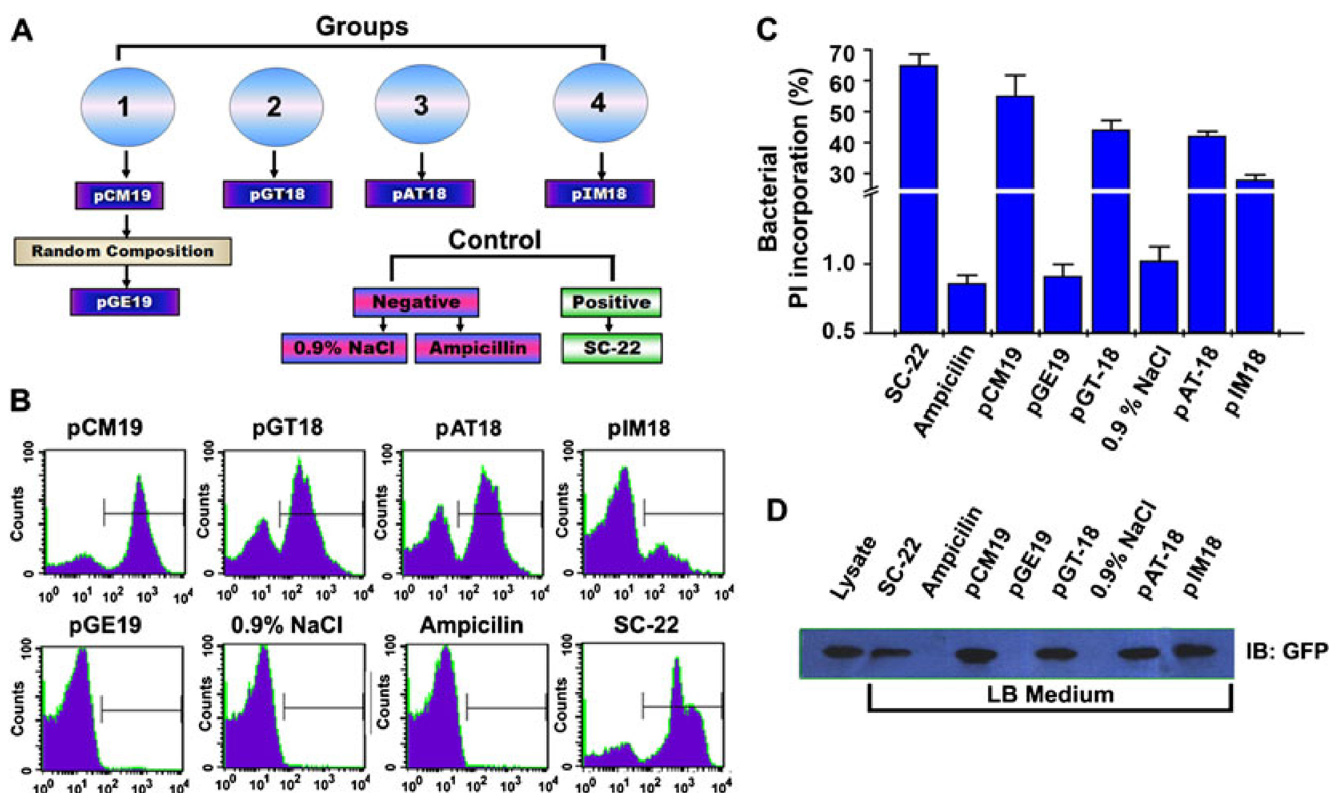

Figure 3. Proteins homologous to hGlyrichin have antibacterial activity. (A) Schematic of hGlyrichin homologous proteins and experimental design. (B) Flow cytometric analysis of bacterial membrane breakage through PI incorporation in *E. coli*. The *E. coli* were cultured in LB media and were collected by centrifugation following 20 min incubation with the indicated solutions in the absence of detergent; peptide concentration is 1000 μg/ml. (C) Flow cytometric analysis of bacterial death through PI incorporation in *E. coli* 20 min post-incubation. Data are expressed as the mean ± SD ($N = 3$). (D) Membrane breakage analysis by western blotting after incubation of *E. coli* (containing pTAT-EGFP) with the indicated peptides and positive and negative controls. The culture medium was collected 1 h after incubation, and western blotting was conducted using anti-GFP antibody. Bacterial whole lysates were used as a positive control for TAT-EGFP expression.

EGFP vector made it possible to observe the integrity of the *E. coli* membrane post-incubation with the antibacterial peptides. To confirm the ability of the peptides to break the bacterial membrane, we incubated pCM19 and the homologous proteins with *E. coli* expressing pTAT-EGFP and determined if GFP leaked into the culture medium. Consistent with the data in Figure 3B and C, GFP protein leakage was detected in the culture medium of *E. coli* that was incubated with pCM19, pGT18, pAT18, or the positive control SC-22. In contrast, GFP proteins were not detected in the culture medium of *E. coli* that was incubated with pGE19, 0.9% NaCl, or ampicillin (Figure 3D). These results confirm that proteins homologous to hGlyrichin kill bacteria by inducing bacterial membrane breakage.

hGlyrichin Does Not Induce Hemolysis *In Vitro* and *In Vivo*

The above data clearly indicate that hGlyrichin unambiguously possesses antibacterial properties. However, it is known that many antibacterial peptides are 'double-edged swords' in that they not only break bacterial membranes but also destroy blood cell membranes (hemolysis). This side effect is a limitation for the use of antibacterial peptides in clinical applications [20]. Therefore, to examine if hGlyrichin induces this side effect, we conducted hemolysis assays. Following the incubation of human blood cells with the pCM19 peptide, the OD570 value was measured and showed that pCM19, similar to the negative control groups that were treated with water or ampicillin, did not induce blood cell dissolution *in vitro* (Table 1). Consistent with the *in vitro* assay, *in vivo* hemolysis assays also demonstrated that pCM19 did not induce the dissolution of mouse peripheral blood cells (Table 2). Therefore, the absence of the hemolytic side effect indicates that hGlyrichin is a potential candidate for antibacterial protein treatments in clinical applications.

Table 1. Incubation of pCM19 with blood cells does not induce hemolysis *in vitro*

Solution	OD570			Mean	SD
	Sample 1	Sample 2	Sample 3		
TritonX-100 (positive control)	4.857	5.979	5.859	5.565	0.502
pCM19 (100 μg/ml)	0.076	0.152	0.103	0.109	0.054
pCM19 (200 μg/ml)	0.116	0.137	0.114	0.122	0.012
pCM19 (500 μg/ml)	0.101	0.096	0.145	0.114	0.006
pCM19 (1000 μg/ml)	0.114	0.124	0.113	0.117	0.007
pCM19 (2000 μg/ml)	0.154	0.131	0.127	0.137	0.002
pCM19 (3000 μg/ml)	0.109	0.128	0.119	0.119	0.001
Normal saline (0.9% NaCl, negative control)	0.113	0.098	0.108	0.106	0.011
Ampicillin (2000 μg/ml)	0.109	0.107	0.085	0.101	0.011

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Table 2. Intravenous injection of CM-19 into mice does not induce hemolysis *in vivo*

Index of blood	Value ($M \pm$ SD), $N = 5$, time (h) after injection					
	Control (saline)		5 mg/kg of CM-19		10 mg/kg of CM-19	
	2 h	5 h	2 h	5 h	2 h	5 h
WBC[a] ($\times 10^9$/l)	8.20 ± 0.53	9.40 ± 1.51	14.53 ± 4.11	9.73 ± 0.61	17.40 ± 5.83	13.73 ± 3.51
RBC[b] ($\times 10^{12}$/l)	8.24 ± 0.79	7.88 ± 0.49	8.63 ± 0.36	7.51 ± 0.75	7.73 ± 0.39	8.15 ± 0.70
HGB[c] (g/l)	132.67 ± 17.93	138.01 ± 26.15	109.33 ± 3.06	98.67 ± 3.06	119.33 ± 11.02	105.33 ± 8.08
HCT[d] (fl)	0.43 ± 0.04	0.41 ± 0.05	0.47 ± 0.03	0.38 ± 0.03	0.40 ± 0.03	0.43 ± 0.02
MCV[e] (g/l)	51.23 ± 0.65	53.40 ± 0.82	50.97 ± 1.17	54.23 ± 1.11	51.60 ± 0.63	52.66 ± 3.31
MCH[f] (pg)	16.23 ± 2.76	17.57 ± 3.46	12.67 ± 0.25	13.23 ± 1.27	15.50 ± 2.21	12.93 ± 0.25
MCHC[g] (g/l)	316.00 ± 50.12	328.67 ± 67.02	234.00 ± 9.17	259.33 ± 19.86	300.67 ± 46.52	246.33 ± 16.62
PLT[h] ($\times 10^9$/l)	1022.67 ± 177.01	869.33 ± 165.85	817.33 ± 261.03	650.66 ± 158.77	874.00 ± 141.48	952.67 ± 111.88

[a]White blood cells.
[b]Red blood cells.
[c]Hemoglobin.
[d]Hematocrit.
[e]Mean corpuscular volume.
[f]Mean corpuscular hemoglobin.
[g]Mean corpuscular hemoglobin concentration.
[h]Platelets.

Discussion

In this study, we show that hGlyrichin, a human gene product, possesses antibacterial activity. The key residues that exert this antibacterial activity are located at amino acid positions 42–60, i.e., the 19 amino acid peptide designated pCM19. We show that hGlyrichin kills the non-pathogenic bacteria *E. coli* and a variety of pathogenic bacteria that includes three MDR bacteria, i.e., MRSA, MDR-PA, and MDR-TB. Furthermore, we show that the mechanism underlying the hGlyrichin killing of bacteria is by the destruction of the bacterial membrane, similar to the well-known antimicrobial peptide SC-22 [18,19]. These findings suggest that hGlyrichin is a potential candidate as a protein antibiotic that fights pathogenic MDR bacteria.

Antimicrobial peptides (AMPs) are ancient and powerful weapons against microbial infection in all forms of life [21]. AMPs have a broad spectrum of antimicrobial activities, and their targets include bacteria, fungi, spirochetes, and viruses [22,23]. To date, more than 1200 AMPs have been deposited in the database (antimicrobial peptide database (APD), http://aps.unmc.edu/AP/main.php/). Most of these AMPs originate from species other than humans. Because AMPs from other species cause hetero-immunological rejection in humans, their clinical applications are limited. The non-immunological rejection of human hGlyrichin is an unparalleled advantage over the AMPs from other species.

Multidrug-resistant bacteria are a tremendous threat to human health or even human life because of the failure of treatments with traditional antibiotics. Therefore, development of novel antibacterial agents, such as antibacterial peptide drugs [24], is an urgent requirement for fighting MDR bacteria [25]. However, the investigation of AMPs to control MDR bacteria are still underway [26,27]. Recent reports have indicated that the innate defense-regulator peptide (IDR-1) [28] and synthetic pheromonicin [29] can selectively kill MRSA, an MDR bacteria. Compared with IDR-1 and pheromonicin, the antibacterial spectrum of pCM19 is broader.

In this study, we identified hGlyrichin as a novel human gene product that possesses antibacterial activity. hGlyrichin can kill a variety of bacteria, including three MDR bacteria, with an efficiency of 100%. We show that all peptides that are homologues of hGlyrichin have antibacterial activity and can penetrate the bacterial membrane. Importantly, hGlyrichin has no side effects, such as hemolysis, *in vitro* or *in vivo*. Therefore, we speculate that hGlyrichin is a promising substitute for traditional antibiotics to fight against the newly emerging MDR bacteria.

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Supporting Information

Supporting information may be found in the online version of this article.

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