Binding Sites on Laminin Receptors as Components for Antibiotics

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Abstract: Bacteria use the receptor-adhesion-like interaction between laminin and the laminin receptor in the process of infection. We determined that bacteria do not interact with the receptor-binding site on laminin which could be expected for the bacterial laminin receptor. Rather, binding occurs via the laminin-binding site on the 67-kDa laminin receptor, which has a function similar to the one the bacterial laminin receptor possesses. This finding has implications for the effective use of antimicrobial peptides.

Keywords: Laminin, laminin receptor, antimicrobial peptide, minimum inhibitory concentration (MIC), prion, binding site.

INTRODUCTION

Laminin is an extracellular glycoprotein that mediates various biological activities including promotion of cell adhesion, growth, migration, differentiation, neurite outgrowth, and tumor metastases [1]. Several specific cell-surface receptors have been reported to be laminin receptors. Laminin receptors are present on the surface of normal and cancer cells and mediate strong attachment of cells to laminin. Tumor cells can invade organ parenchyma by attaching to the basement membrane via laminin receptors in the initial step of metastasis [1, 2]. Laminin receptors also could act as potential receptors for cellular prion proteins which are cell membrane glycoproteins [3]. When the cellular prion protein is internalized by clathrin-coated pits according to an endocytic pathway, a laminin receptor could be responsible for making the connection between the surface-anchored cellular prion protein and the clathrin. Laminin receptors also might be involved in the conversion of cellular prion proteins to their pathogenic isoform, scrapie prion proteins, which are thought to occur in endosomes, lysosomes, or endolysosomes of the endocytic pathway in the life of prions [4].

The laminin receptor family is highly conserved in a wide spectrum of eukaryotic cells, including yeast [5]. Bacteria also have laminin receptors on the cell surface similar to eukaryotic laminin receptors [5-8]. The receptor-adhesion-like interaction between laminin and the laminin receptor is thought to be the first step in the process of protozoan and bacteria infection [6-10]. Therefore, we focused on each interactive binding site of laminin and a laminin receptor and performed experiments to find antimicrobial potential in peptides derived from the laminin and laminin receptor binding sites. Experiments were also done to determine whether antimicrobial potential would be found in peptides derived from the laminin receptor-binding site on a cellular prion protein. As a result of the experiments, we discovered that it is not the laminin receptor-binding sites on laminin and the cellular prion protein which could possibly interact with the bacterial laminin receptor, but, rather, it is the laminin-binding site on the laminin receptor. This has a function similar to the one the bacterial laminin receptor possesses and has the potential to become an effective component for an antimicrobial peptide.

MATERIALS AND METHODS

Peptide Synthesis

The peptides were synthesized by 9-fluorenylmethoxycarbonyl solid phase synthesis method using Rink amide resin on an automated solid-phase peptide synthesizer (APEX 396, Advanced ChemTech, USA). After synthesis, the peptides were cleaved with TFA cocktail reagent (80% TFA, 6% 1,2-Ethanedithiol, 2% m-cresol, and 12% thioanisole) for 3 hours, and then precipitated with cold ether and harvested using centrifugation. The cleaved peptides were purified to over ≥95% purity by reverse-phase high-performance liquid chromatography on a C_{18} column.

Bacteria

The gram positive bacteria used were *S. aureus* IFO12731 and *S. aureus* COL (methicillin-resistant *S. aureus*, MRSA). The gram negative bacteria used were *E. coli* IFO3972 and *P. aeruginosa* ATCC 10145. These bacterial strains were provided by the University of Tokushima.

Preparation of Bacterial Suspension

Bacterial strains were pre-incubated in a Luria-Bertani broth [1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl, pH 7.0] for 18 hours at 37℃ until they reached the stationary-phase. The cell suspensions were diluted with Mueller-Hinton broth (Difco) until they were adjusted to approximately 2.0×10^6 cells/ml. Then, the suspensions were kept in ice and used within the hour.

Antimicrobial Assay

The minimum inhibitory concentrations (MICs) of the synthesized peptides were evaluated by following the microdilution method. The peptide-diluted series were prepared with Mueller-Hinton broth (Difco) in a 96-well plate, to

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which the bacterial suspension was added (10^6 cells/ml in the total broth volume of 300 μL) and incubated at 37℃ for 24 h. The MICs were then evaluated by visual inspection.

RESULTS AND DISCUSSION

In this study, the laminin receptor-binding site on laminin, the laminin-binding site on the laminin receptor, and the laminin receptor binding site on the cellular prion protein are termed the laminin peptide, the laminin receptor peptide, and the prion peptide respectively. Most of these peptides were difficult to test in the antimicrobial assay because of their water-insolubility, so they were combined with a nuclear localization signal (NLS), which we have previously studied in the course of our peptide antibiotic research [11]. Peptide sequences corresponding to the laminin and laminin receptor peptides are shown in Table **1**. The laminin peptides were derived from pigtail monkey (*Macca nemestrina*) cells [12] and these amino acid sequences are highly conserved in mammalian cells. The laminin receptor peptides were derived from the highly conserved sequence, the palindromic sequence LMWWML, in multiple alignment of the region spanning aa 161-180 (numbering refers to the human sequence), the laminin-binding site of the 67-kDa laminin receptor, and its homologues in various eukaryotes [5]. A peptide sequence corresponding to the prion peptide is shown in Table **3**. The prion peptide is derived from the cellular prion protein aa 144-179 which has been identified as a direct interaction domain with laminin receptor aa 161-179 in the yeast two-hybrid system [3]. All types of peptides were combined to the N or C terminals of three kinds of NLS peptides, NLS_1 (p54): RIRKKLR, NLS_2 (SV40 Large T antigen): PKKKRKV, and NLS_3 (Pax-QNR): LKRKLQR [13]. The MICs of the peptides were then evaluated.

The results of MICs for the synthesized peptides based on the laminin and laminin receptor peptides are summarized in Table **2**. The evaluated peptides are classified into four types: the NLS peptide (NLS), the laminin peptide linked to an NLS C terminus (NLS+Ln), the laminin receptor peptide linked to an NLS C terminus (NLS+LR), and the laminin receptor peptide linked to an NLS N terminus (LR+NLS). NLS on their own had no antimicrobial activity for all the bacteria tested and their MICs were over 100 μg/ml. NLS_1+Ln_1 and NLS_1+Ln_2 had no activity for any of the bacteria either (MICs of >100 μg/ml). However, the NLS_1+LR_1 demonstrated remarkably strong antimicrobial activity and its MICs for all the bacteria were in the region from 6.3 μg/ml to 25 μg/ml. Even when the LR_1 was linked to an NLS_1 N terminus (LR_1+NLS_1) or another NLS C terminus (NLS_2+LR_1), strong antimicrobial activity was still demonstrated. Moreover, to investigate if this strong antimicrobial activity is also present in other laminin receptor peptides, six types of laminin receptor peptides derived from various eukaryotes linked to an NLS C terminus (NLS_3+LR_1, NLS_3+LR_2, NLS_3+LR_3, NLS_3+LR_4, NLS_3+LR_5, NLS_3+LR_6) were evaluated for MICs. As a result, it was confirmed that the highly efficient antimicrobial function is commonly inherent in various kinds of laminin receptor peptides. The MICs of these peptides were in the region from 3.1 μg/ml to 50 μg/ml. The laminin peptide is supposed to bind to a bacterial laminin receptor much like a key fitting into a lock. Unlike the case of the laminin peptide, the laminin receptor peptide and bacterial laminin receptor cannot bind just as two locks cannot come together. Ordinarily, the former, the laminin peptide, would receive attention as a candidate for a component in an antimicrobial peptide. However, our finding clarified a surprisingly high potential in the laminin receptor peptide (the latter) for antimicrobial function.

The laminin receptor has also been known to act as a receptor for the cellular prion protein [3]. The cellular prion protein-binding site on a laminin receptor is thought to be the same conserved sequence, LMWWML, as the laminin-binding site on a laminin receptor [4]. We carried out the

Table 1. Sequences of Laminin Peptides and Laminin Receptor Peptides

Peptide[a]	Sequence	Organism of origin
Laminin peptide (Laminin receptor-binding site on laminin)		
Ln_1	YIGSR	*Macaca nemesterina*
Ln_2	RGD	*Macaca nemesterina*
Laminin receptor peptide (Laminin-binding site on laminin receptor)		
LR_1	LMWWML	*Homo sapiens*
LR_2	LIWYLL	*Saccharomyces cerevisiae (mt)*[b]
LR_3	FFYMVI	*Acanthamoeba castellanii (mt)*
LR_4	VVYWLL	*Haloarcula marismortui*
LR_5	CLFWLL	*Arabidopsis thaliana*
LR_6	LMWWLL	*Drosophila melanogaster*

[a] Ln_1 and Ln_2: the laminin peptides (Laminin receptor-binding site on laminin) [12], LR_1: the laminin receptor peptide (Laminin-binding site on 67-kD laminin receptor). $LR_{2\text{-}6}$: homology region of LR_1 in various eukaryotes [5].
[b] *mt,* mitochondrial protein.

Table 2. MICs for the Laminin and Laminin Receptor Peptides Linked to NLS

Sequence	Composition[a]	MIC (μg/ml)			
		S. aureus IFO12732	*S. aureus* COL (MRSA)[b]	*E. coli* IFO3972	*P. aeruginosa* ATCC 10145
RIRKKLR	NLS_1	>100	>100	>100	>100
PKKKRKV	NLS_2	>100	>100	>100	>100
LKRKLQR	NLS_3	>100	>100	>100	>100
RIRKKLR YIGSR	$NLS_1 + Ln_1$	>100	>100	>100	>100
RIRKKLR RGD	$NLS_1 + Ln_2$	>100	>100	>100	>100
RIRKKLR LMWWML	$NLS_1 + LR_1$	12.5	12.5	25	6.3
PKKKRKV LMWWML	$NLS_2 + LR_1$	12.5	50	12.5	6.3
LKRKLQR LMWWML	$NLS_3 + LR_1$	3.1	6.3	6.3	6.3
LKRKLQR LIWYLL	$NLS_3 + LR_2$	6.3	12.5	12.5	25
LKRKLQR FFYMVI	$NLS_3 + LR_3$	3.1	25	12.5	50
LKRKLQR VVYWLL	$NLS_3 + LR_4$	3.1	6.3	10.7	6.3
LKRKLQR CLFWLL	$NLS_3 + LR_5$	6.3	50	50	50
LKRKLQR LMWWLL	$NLS_3 + LR_6$	3.1	6.3	22.3	50
LMWWML RIRKKLR	$LR_1 + NLS_1$	3.1	12.5	6.3	6.3

[a] NLS_1, NLS_2, and NLS_3 are derived from p54, SV40 Large T antigen, and Pax-QNR respectively [13].
[b] MRSA, methicillin-resistant *S. aureus.*

Table 3. MICs for the Prion Peptide Linked to NLS

Sequence	Composition[a]	MIC (μg/ml)	
		S. aureus IFO12732	*E. coli* IFO3972
PKKKRKV DYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDC	NLS_2 + Pr	>280	>280

[a] Pr; the prion peptide (LR-binding site of cellular prion protein$^{144\text{-}179}$) [3]. NLS_2 (SV40 Large T antigen): PKKKRKV.

antimicrobial assay for the laminin receptor-binding site on the cellular prion protein (the prion peptide) linked to NLS (NLS_2+Pr), but no antimicrobial function was found (MICs of >280μg/ml). These results are shown in Table **3**.

With these results, it was demonstrated that only the peptides based on the laminin receptor, NLS+LR and LR+NLS, possess a significant antimicrobial function among the peptides tested, NLS, NLS+Ln, NLS+LR, LR+NLS, and NLS+Pr. In addition to traditional design methods which are based on the interaction between a ligand and a receptor, our unique design method of using a receptor with a receptor is another method which could lead to the development of new antibiotics.

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