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Short Communication

Membrane anchoring of a curvature-inducing peptide, EpN18, promotes membrane translocation of octaarginine

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Abstract

EpN18 is a curvature-inducing peptide, which loosens lipid packing upon interaction with the cell membrane, and facilitates cell-membrane penetration by arginine-rich cell-penetrating peptides, including octaarginine (R8). In the present study, we conjugated the N-terminal of EpN18 with a pyrenebutyryl (pBu) moiety, which acts as an anchoring unit that increases membrane interactions. Enhanced lipid-packing loosening and cytosolic translocation of R8 were observed by the pBu anchoring of EpN18.

Keywords

curvature-inducing peptide; octaarginine; cell membrane translocation; pyrenebutyric acid; lipid packing loosening

Abbreviations

R8, octaarginine

pBu, pyrenebutyryl

pBu-OH, pyrenebutyric acid

HPLC, high-performance liquid chromatography

MALDI-TOFMS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry

FITC, fluorescein isothiocyanate

PI, propidium iodide

CLSM, confocal laser scanning microscopy

α-MEM, α-minimum essential medium

di-4-ANEPPDHQ, 2-hydroxy-3-[2-[(2-hydroxyethyl)dimethylaminio]ethyl]-4-[2-[6-(dibutylamino)-2-naphthyl]ethenyl]pyridinium dibromide

GP, generalized polarization

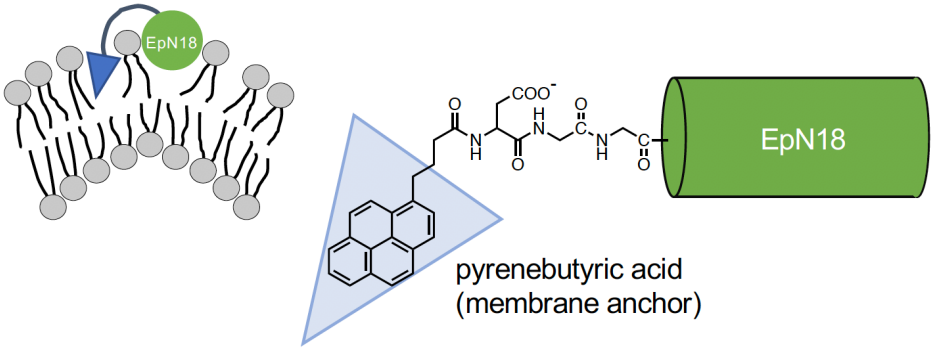
ROI, region of interest

CD, circular dichroism

POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine

Graphical Abstract



Epsin-1 protein is involved in clathrin-coated pit formation during clathrin-dependent endocytosis.[1] Moreover, this protein belongs to the family of proteins known to induce membrane curvature.[2] Our group previously reported that a peptide corresponding to the N-terminal 18 residues of epsin-1 (EpN18: XSTSSLRRQXKNIVHNYS-amide, X = norleucine) can induce positive membrane curvature and loosen lipid packing.[3-5] EpN18 forms an amphiphilic helical structure that interacts with the membrane. Insertion of the hydrophobic face pushes the surrounding lipids, thereby resulting in the expansion of the membrane surface area on the side of peptide insertion and eventually, positive curvature (Figure 1A). Furthermore, we found that translocation of octaarginine, a prototypical cell-penetrating peptide with an ability to transport exogenous bioactive molecules into cells, was promoted in response to the increment in membrane curvature and its concomitant loosening of lipid packing.[3-5] This exemplifies the practical importance of membrane structural alteration as a tool in manipulating cellular function.

A relatively high concentration (i.e., 20–40 μM) of EpN18 is required to produce a significant effect on the membrane structure.[3, 4] On the other hand, epsin-1 protein is known to interact with phosphatidylinositol 4,5-bisphosphate in the inner leaflet of the cell membrane and helps in recruiting this protein to the membrane. This aids the insertion of the N-terminal segment and the induction of membrane curvature.[2] EpN18 does not have a mechanism to recruit itself to the membrane. If some anchoring moiety is attached to EpN18, EpN18 can effectively interact within the membrane to affect the lipid-packing states (Figure 1B). The present study was designed to analyze the validity of the aforementioned strategy in facilitating membrane anchoring of the EpN18 peptide in an effort to enhance its lipid-packing loosening ability.

A pyrenebutyryl (pBu) moiety was employed as the anchoring unit. In addition to its hydrophobic character, we previously found that pyrenebutyric acid (pBu-OH) also induces positive membrane curvature and lipid-packing loosening.[4] We used a DGG sequence linker to conjugate the pBu moiety and EpN18 peptide (Figure 1C). Aspartic acid in the liner has a negative charge in its side chain under physiological conditions, which may help to hold the pBu moiety at a relatively shallow position in the membrane.[6] The thermal motion of the pBu moiety in the membrane may introduce steric interference in the surrounding lipids and help to loosen the lipid packing. The DGG segment is presumed to work as a flexible linker that links both pBu and EpN18 segments without hampering their respective properties. The lack of the aspartic acid in the linker may allow the pBu moiety to travel to more depth from the membrane surfaces. Therefore, a GGG

linker was also employed to evaluate the effect on memrane integrity and lipid-packing loosening.

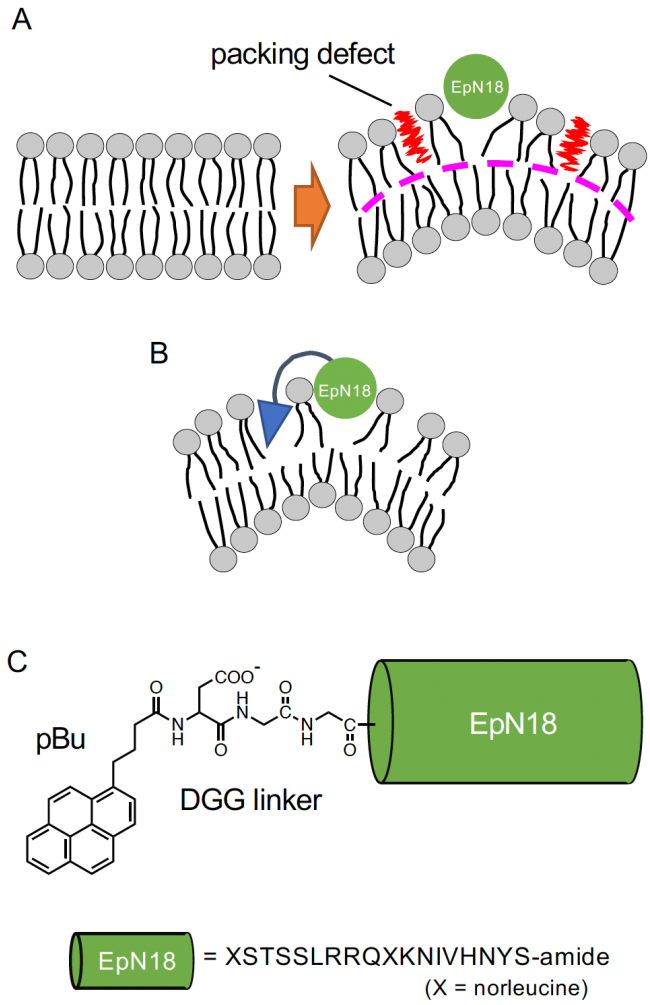


Figure 1. Conceptual illustration of this study. (A) Lipid packing of the cell membranes without curvature (left). Interactions between the membrane and EpN18 (green) yield positive membrane curvature and lipid-packing defect (red) (right). (B) Membrane anchoring with a pyrenebutyryl (pBu) moiety (blue) enhances the membrane interactions of EpN18. (C) Structure of pBu-DGG-EpN18.

Both peptides (pBu-DGG-EpN18 and pBu-GGG-EpN18) were prepared using Fmoc (9-fluorenylmethoxycarbonyl) solid phase peptide synthesis in a Rink amide resin.[7, 8] After construction of the peptide chain on the resin, pBu-OH was introduced to the N-termini of the peptides and was followed by deprotection of the side-chain-protecting groups. Thereafter, these peptides were detached from the resin (using a mixture of trifluoroacetic acid and 1,2-ethanedithiol (95:5)) and then purified by high-performance liquid chromatography (HPLC). The mass of these products was then confirmed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOFMS, Table S1).

Moreover, we evaluated the effect of pBu-DGG-EpN18 and pBu-GGG-EpN18 on the cellular penetration of fluorescently labeled (fluorescein isothiocyanate, FITC) octaarginine (FITC-R8). Furthermore, it is believed that FITC is a model of low-molecular weight drugs, which need to be delivered directly to the cells. Delivery of FITC, with the help of octaarginine, into the cell, allows the signal to be distributed throughout the cell. Therefore, the cell count with diffuse cytosolic FITC signals was compared to analyze the membrane translocation efficacy of these peptides (Figure 2).

Before analyzing the detailed modes of the membrane interactions of these peptides, the concentration range of the peptides (i.e., the concentration at which membrane integrity was retained following coincubation with FITC-R8 (10 μM)) was estimated using propidium iodide (PI), and 10 μM FITC-R8 was employed in subsequent studies to evaluate the effect of the pBu moiety on lipid packing and promotion of FITC-R8 internalization. PI is a nuclear staining dye that cannot permeate cells through intact cell membranes[9]; however, once the cell membrane is damaged, the dye can enter the cells and accumulate in the nucleus. Therefore, membrane integrity can be analyzed following PI treatment using confocal laser scanning microscopy (CLSM) (Figure S1A). While marked nuclear staining was observed in cells treated with 10 μM pBu-DGG-EpN18, 4 μM pBu-DGG-EpN18 yielded marginal PI signals in the nucleus. pBu-GGG-EpN18 exhibited higher membrane interaction and induced remarkable membrane rupture compared with pBu-DGG-EpN18. Significant nuclear staining was observed after treatment with 1 μM pBu-DGG-EpN18. To avoid notable PI signals in the nucleus, the concentration had to be reduced to 0.5 μM. Thus, 4 μM pBu-DGG-EpN18 and 0.5 μM pBu-GGG-EpN18 were employed in subsequent analyses.

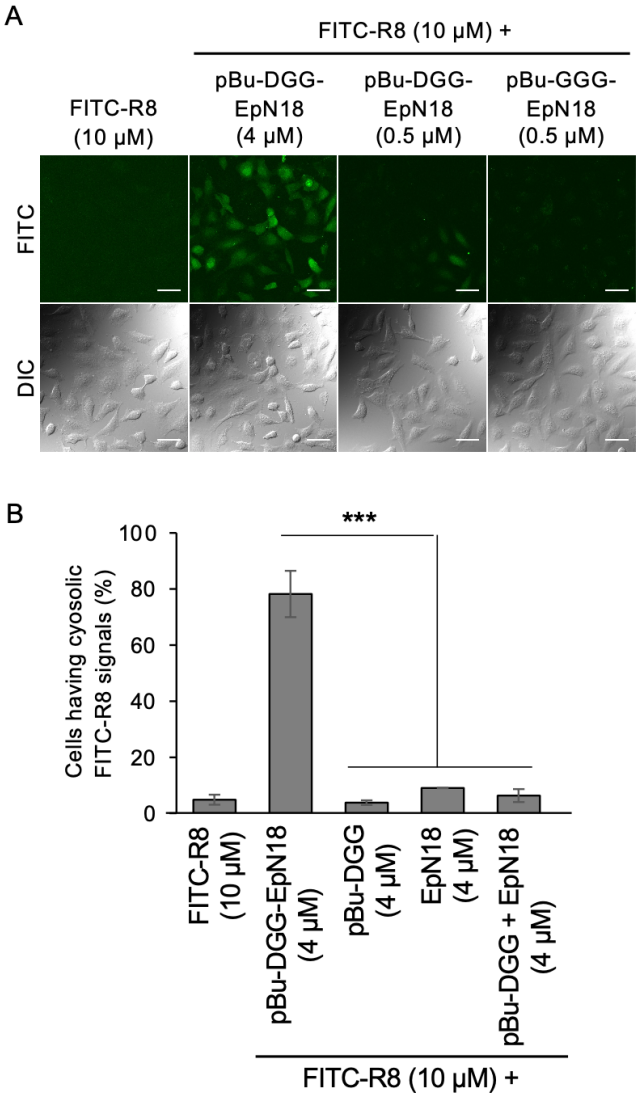


Figure 2. (A) Confocal laser scanning microscopy observation of the cells treated with FITC-R8 (10 μM) in the presence of pBu-DGG-EpN18 or pBu-GGG-EpN18 in serum free medium [α-MEM(–)] for 5 min. Scale bars, 50 μm. (B) Percentage of cells with diffuse cytosolic FITC-R8 signal after treatment with FITC-R8 (10 μM) in the presence of pBu-DGG-EpN18 (4 μM), pBu-DGG (4 μM), EpN18 (4 μM), and a mixture of pBu-DGG (4 μM) and EpN18 (4 μM), respectively, in α-MEM(–)] for 5 min. Tukey's test: ***, $p < 0.001$.

Cells were treated with 10 μM FITC-R8 in the presence of pBu-DGG-EpN18 or pBu-GGG-EpN18 for 5 min in serum-free α-minimum essential medium [α-MEM(–)]. Cells that were treated with FITC-R8 alone exhibited a marginal level of cytosolic FITC signal (Figure 2A). In contrast, coincubation with 4 μM pBu-DGG-EpN18 yielded marked diffuse cytosolic FITC expression. Treatment with pBu-GGG-EpN18 (0.5 μM) demonstrated less cytosolic fluorescence, and when the percentages of cells with cytosolic FITC-R8 signals were enumerated, FITC-R8 alone yielded cytosolic FITC-R8 signals in only 5% of the cells (Figure 2B). This frequency increased to approximately 80% in the presence of 4 μM pBu-DGG-EpN18. In contrast, pBu-DGG (lacking the EpN18 segment) alone, EpN18 alone, or a mixture of EpN18 and pBu-DGG yielded only a marginal increase in the number of cells with cytosolic FITC-R8 labeling. Moreover, treatment with 0.5 μM pBu-GGG-EpN18 yielded cytosolic FITC-R8 signals in approximately 15% of cells (Figure S1B), suggesting the importance of the Asp residue in the DGG linker in promoting the membrane translocation of FITC-R8. The advantage of pBu-DGG-EpN18 may come from its lower cytotoxicity compared with pBu-GGG-EpN18, which allows the use of 4μM on the treatment with FITC-R8. Use of 0.5 μM pBu-DGG-EpN18, as in the case of pBu-GGG-EpN18, yielded poor cytosolic distribution of FITC-R8 (Figure 2A). Considering the aforementioned results, we evaluated the characteristics of pBu-DGG-EpN18 in more detail.

The effect of pBu-DGG-EpN18 on loosening membrane packing was further analyzed in terms of the generalized polarization (GP) of the cell membrane using an environment-sensitive fluorescent probe (di-4-ANEPPDHQ) (Figure 3A, B). This dye has a 60-nm spectral blue shift between the disordered and ordered bilayer phases.[10] This allows a quantitative assessment of membrane order by calculating a ratiometric analysis of the fluorescence intensity recorded in two spectral channels, known as a GP value.[11] A decrease in the GP values indicates an increase in polarity or lipid-packing loosening.

The cells were treated with 4 μM pBu-DGG-EpN18 in the presence of di-4-ANEPPDHQ, and the difference in GP value (ΔGP) in peripheral areas of the cells, 10 min before and after peptide addition, was analyzed as previously reported (Figure 3A). This analysis revealed an average ΔGP of −0.05 (Figure 3B), whereas the same evaluation using 4 μM EpN18 yielded no significant decrease in the GP value compared with that in the no treatment control. Treatment with pBu-DGG (4 μM) and a mixture of pBu-DGG and EpN18 (4 μM each) marginally decreased the GP values. This suggests that pBu-DGG-EpN18 induces more significant lipid-packing loosening than the other constructs, which

is likely because of the changes in the cross-linking activity of these peptides.

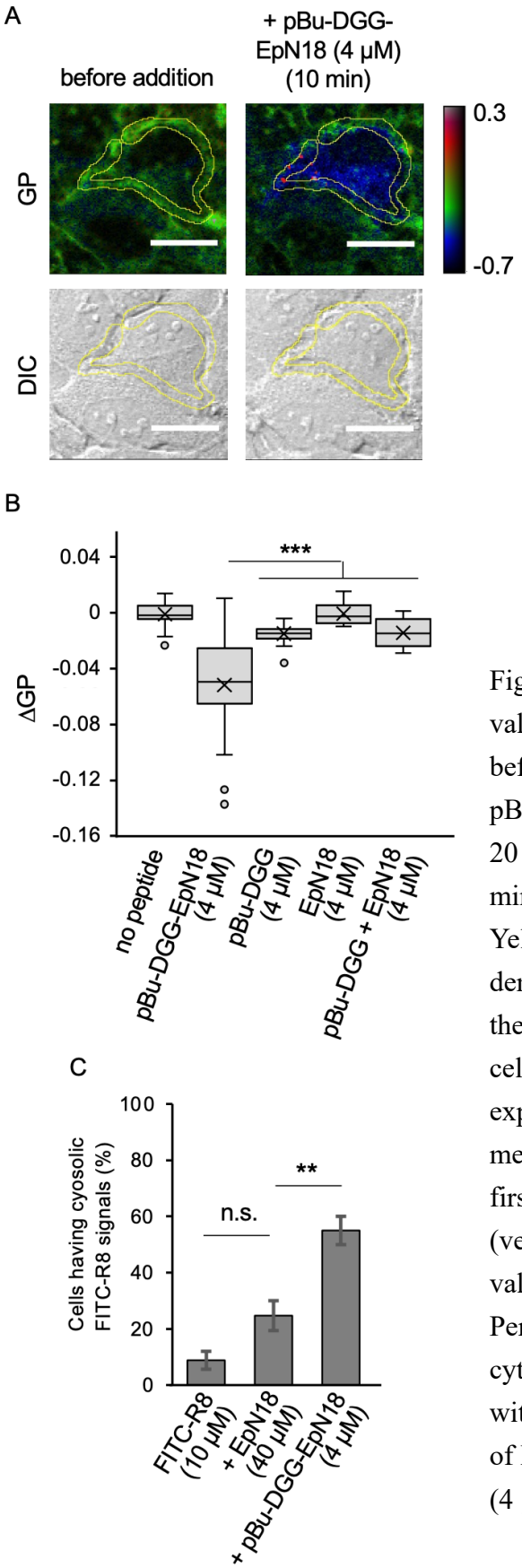


Figure 3. (A) Pseudocolor images of GP values and DIC images obtained 3 min before and 10 min after treatment with pBu-DGG-EpN18 (4 μM). Scale bars, 20 μm. (B) ΔGP of the membranes 10 min after treatment with peptides. Yellow lines in (A) are examples of demarcating regions of interest (ROIs) at the cell periphery. Box plots include 30 cells from three independent experiments. This image shows the median (horizontal bars), average (×), first and third quartile (boxes), range (vertical bars), and outlier (circles) values. Tukey's test: ***, $p < 0.001$. (C) Percentage of cells showing a diffuse cytosolic FITC-R8 signal after treatment with FITC-R8 (10 μM) in the presence of EpN18 (40 μM) or pBu-DGG-EpN18 (4 μM) in α-MEM(–) for 5 min.

We previously reported that EpN18 has the ability to loosen lipid packing and promote translocation of FITC-R8 through the cell membrane; however, at that time, 40 μM EpN18 (10× the concentration used in this study) was needed to induce these changes in the cell. Therefore, we employed 40 μM EpN18 and 4 μM pBu-DGG-EpN18 and compared their ability to facilitate FITC-R8 translocation. This produced a more than two-fold increase in the number of cells with diffuse FITC-R8 labeling, following treatment with 10 μM FITC-R8 in the presence of 4 μM pBu-DGG-EpN18 for 5 min compared with the number of cells obtained using 40 μM EpN18 (Figure 3C).

Increased membrane interactions following pBu-mediated anchoring of the EpN18 peptide may be reflected in the circular dichroism (CD) spectrum (Figure 4). EpN18 was reported to adopt a helical structure in the presence of liposomes produced using POPC (=1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine):POPS (=1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine) (50:50) where the negatively charged lipid was crucial in adopting the helical structure.[3] CD spectra of the EpN18 peptides in phosphate-buffered saline (PBS) or in the presence of POPC/POPS (75:25) liposomes (reduced POPS content resulted in reduced adoption of the helical confirmation in EpN18 peptides) were not suggestive of typical helical conformation (Figure 4, blue filled diamond). Moreover, pBu-DGG-EpN18 peptides did not form marked helical structures in the buffer; however, these peptides did adopt a distinct helix-like structure in the presence of POPC:POPS (75:25) LUVs, thereby yielding a pair of minima around 208 and 222 nm[12] and a shoulder peak at around 245 nm. This is presumably the result of pBu inclusion[13] (Figure 4, orange open circle). Collectively, these data suggest increased interaction between the membrane and peptide in the presence of the pBu moiety.

In conclusion, this study highlights the validity of the aforementioned strategy in enhancing lipid-packing loosening and cell penetration by conjugated peptides (FITC-R8). Herein, we demonstrated that the addition of a pBu-based anchor improves the interactions between the EpN18 peptide and the cell membrane. Further evaluation and optimization of these curvature-inducing peptides, linkers, and anchoring moieties may lead to more effective systems for manipulating lipid packing and membrane structure. These trials are ongoing in our laboratory.

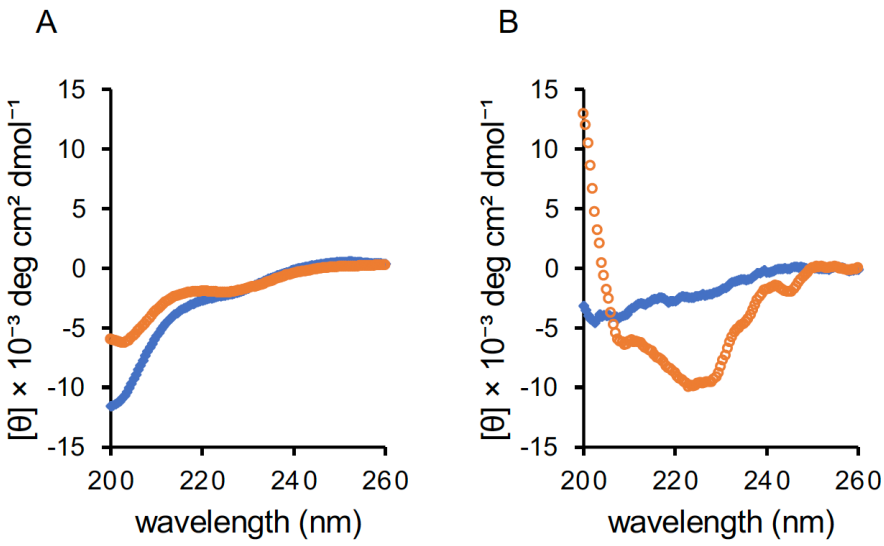


Figure 4. CD spectra for EpN18 (blue filled diamond) and pBu-DGG-EpN18 (orange open circle) (20 μM each) in the absence (A) and presence (B) of POPC: POPS (75:25) LUVs (lipid concentration, 200 μM) in PBS.

Acknowledgments

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Declaration of Interest

The authors declare no conflicts of interest associated with this manuscript.

Supplementary Material

Materials and Methods.

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