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Understanding Lipid Recognition by Protein-Mimicking Cyclic Peptides

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Abstract

This paper describes our investigation of the structural determinants of a designed cyclic peptide (cLac, cyclic peptide mimicking lactadherin)¹ for phosphatidylserine (PS) recognition. A highly efficient strategy that takes advantage of the native chemical ligation (NCL) chemistry has been developed for the synthesis and labeling of cyclic peptides in general. A scanning of the cLac peptide revealed a sophisticated model for PS binding, in which the peptide scaffold assembles multiple polar residues to balance the desolvation and electrostatic interactions (salt bridge and hydrogen bonding) to achieve lipid selectivity. The results suggest that cLac effectively mimics the membrane binding mechanism of the parent protein lactadherin.

Keywords

cyclic peptide; native chemical ligation; phosphatidylserine; lactadherin; cLac

Introduction

As a defining feature of a living cell, the plasma membrane serves as a gate for material exchange between the cell and its environment. Of equal importance, it also mediates numerous signaling events from outside stimuli to internal response. It is becoming increasingly clear that membrane lipids are not mere bystanders in material exchange and signaling. Instead, they play active roles by directly functioning as receptors or by recruiting relevant proteins and controlling the spatial organization of these protein molecules.² Therefore, the spatiotemporal distribution of lipids underlies essentially all functions of the membrane. Recent mass spectrometry analysis of the lipidome (the collective population of lipids) suggests over 1000 distinct membrane lipids in a mammalian cell.³ Given the complexity of the lipidome, it is highly desirable to have affinity ligands for individual lipids so that their spatiotemporal distribution can be tracked and correlated to biological phenotypes.

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We have undertaken a program to develop affinity ligands for membrane lipids. Inspired by peptide natural products such as cinnamycin (Figure 1), which specifically binds phosphatidylethanolamine (PE),^{4,5} we hypothesized that properly designed cyclic peptides could serve as effective receptors of membrane lipids.⁶ Indeed, our previous work shows that cyclic peptides that mimic the lipid-binding protein lactadherin display selective binding to phosphatidylserine (PS).¹ PS is known to exclusively reside in the inner leaflet of healthy cells and its externalization accompanies important processes including cell apoptosis and blood coagulation. By tracking the surface exposed PS, a lactadherin mimicking peptide (cLac) with fluorophore labeling effectively stains apoptotic cells as the parent protein does. In this contribution, we report a structure-activity study of the cLac peptide in terms of lipid binding. Importantly, we also describe a highly efficient strategy for cyclic peptide synthesis and labeling that is based on the native chemical ligation chemistry.

Cyclic peptide synthesis

Most previous reports on cyclic peptide synthesis utilized a side chain-immobilized Asp or Glu residue on resin (Figure 2a).^{7,8} Selective deprotection of the main chain COOH group after peptide synthesis enables peptide cyclization with common amide bond formation conditions (e.g., the use of PyBOP). While this method applies to various peptide lengths and sequences, side products are often seen because of epimerization of the C-terminal residue, incomplete cyclization, and formation of peptide dimers. We envisioned that native chemical ligation (NCL) chemistry,⁹ which takes advantage of the orthogonal reactivity of an N-terminal cysteine and a C-terminal thioester, could afford a more efficient protocol for cyclic peptide synthesis. Indeed, the pioneering work by Benkovic and associates showed that cyclic peptides could be generated in bacterial cells with intein-mediated formation of thioesters.¹⁰ A similar strategy has later been adopted by Kritzer et al. to create cyclic peptides in yeast cells.¹¹ Importantly, NCL-cyclized peptides carry a free Cys side chain, which provides a handle for peptide labeling with various thiol-reactive fluorophores that are commercially available.

The synthesis of peptide thioesters via the Fmoc chemistry had been difficult until recently. Elegant work from the Dawson lab showed that solid supports functionalized with 3, 4-diaminobenzoic acid (named as Dawson Dbz resin) allows facile synthesis of peptides with an N-acyl urea structure on the C-terminus, which upon treatment with thiophenol (or derivatives) transforms into a thioester (Figure 2b). The *in situ* generated thioesters can readily go through intramolecular NCL to yield cyclic peptides. Our synthesis began with the commercially available Dawson Dbz resin. We chose to install a cysteine residue in one of the linker regions of the original cLac sequence (Figure 3a); this linker region was shown to better tolerate variations than the other.¹ The standard protocol for Fmoc peptide synthesis was implemented on an automated peptide synthesizer to assemble the linear precursor of the cLac variants. At the end of the synthesis, the resin was treated with para-nitrophenyl chloroformate to afford the urea activated C-terminal carbonyl group. A Boc protected amino acid was used for the last residue to avoid modification of the N-terminus. The peptides were cleaved off resin with reagent K and purified on prep HPLC with a C-18 column. Typically an isolated yield around 60% was achieved for the linear precursor peptides. The peptide cyclization was initiated by the addition of 4-mercaptophenylacetic

acid to form the thioester intermediate. Under optimized conditions (see the experimental section), the linear precursors completely cyclized over a period of 4 hours and the desired cyclic products were isolated via HPLC at 60–70% yield. The less than perfect recovery yield is largely due to the mechanical loss during purification as the cyclization usually proceeds cleanly without giving any significant side products (Figure 2c). Taking advantage of the free cysteine side chain, we labeled all cLac variants with the thiol-reactive fluorescein-5-maleimide in DMF containing 2% NMM (N-methylmorpholine) as a weak base. The stronger base DIPEA (N, N-diisopropyl ethylamine) resulted in hydrolysis of the succinimide moiety after the dye conjugation.

Lipid binding analysis

The sequences of the cLac variants used in this study are summarized in Figure 3b. For convenience of discussion, we refer to the Gly-to-Cys variant of cLac generated by NCL cyclization as cLac-wt. In order to dissect the key structural determinants of PS binding by the cLac peptides, we have mutated the polar residues individually to an Ala. We have also prepared two cLac variants that incorporate one or two additional cationic residues to assess the significance of charge-charge attractions in cLac-membrane association. The peptide variants were first analyzed with a FRET (fluorescence resonance energy transfer) assay, the results of which were further corroborated with a fluorescence polarization assay.

Peptides without the fluorophore label were used in the FRET assay, for which large unilamellar vesicles (LUVs, 100 nm in diameter) were prepared with POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) as the matrix lipid. All vesicles include 5% POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine) conjugated with a dansyl label, which serves as a FRET acceptor. Upon peptide binding onto membranes, FRET occurs from the Trp residue of the peptide to the vesicle displayed dansyl groups. Similar to the original cLac peptide,¹ the NCL-generated cLac-wt displays PS selective membrane binding, indicating that the added cysteine side chain does not interfere with cLac binding onto membranes. Also consistent with our previous report, the dansyl fluorescence increase in the FRET assay correlates well with the percentage of PS and therefore the amount of membrane-bound peptides. For example, with an excess amount of peptides, the dansyl fluorescence increase for the vesicles with 20% PS is roughly four times as much as the vesicles with 5% PS (Figure 3c). Therefore, we use the dansyl fluorescence increase to assess the relative membrane binding propensity of the cLac variants.

Interestingly, the R/A mutant failed to elicit significant increases of dansyl emission against all three different types of vesicles, indicating that the Arg residue plays a critical role in the cLac-membrane interaction. In contrast, the D/A mutant afforded a similar degree of membrane association as cLac-wt against vesicles with 20% PS. However, the D/A mutant elicited significant dansyl emission against vesicles containing 0% and 5% PS as well, albeit at a lower intensity than against vesicles with 20% PS. These data suggest that the Asp residue makes a critical contribution to the PS selectivity (over PC) of cLac-wt. Mutating the Gln residue to Ala resulted in a lipid selectivity profile similar as that of the D/A mutant with a slightly less favorable membrane partition. Surprisingly, mutating the His residue to

Ala completely abolished the PS selectivity of cLac as a result of both elevated binding to PC-only vesicles and reduced binding to vesicles with 20% PS.

The membrane binding properties of the cLac variants were further validated with a fluorescence polarization assay. In contrast to the FRET assay where an excess amount of peptides were titrated into lipid vesicles, the fluorescence polarization assay required that the cLac peptides remained at a low concentration, into which increasing amounts of lipid vesicles were titrated and the anisotropy values recorded (Figure 4). The results of the polarization assay largely agree with those of the FRET assay. Specifically, the R/A mutant yielded no membrane binding in both the presence and absence of PS; the D/A and Q/A mutants showed significant association with PC-only vesicles, affording compromised selectivity for PS. Surprisingly, the H/A mutant gives little binding to either type of vesicles under our experimental conditions. It is possible that the fluorescein label might have changed the structural preferences of this peptide in the unbound state. Further experiments are needed to understand the chemical basis of this observation.

The binding data presented above are consistent with the PS binding model (Figure 5) we previously proposed for lactadherin, the PS binding protein¹² that the cLac peptide was designed to mimic. According to this model generated through molecular docking experiments, the Arg residue forms a double hydrogen-bonded salt bridge with the phosphate group of PS. Arg is well known to give strong binding with a phosphate group.^{13,14} Elimination of this strong interaction is likely to explain the complete loss of membrane binding of the R/A mutant. The His residue appears to engage in a polar interaction with the carboxylate moiety of PS through either a hydrogen bond or a salt bridge, depending on its ionization status. Given that the carboxylate group is unique to PS while absent in PC, elimination of this PS carboxylate-His interaction is expected to abolish the PS selectivity of cLac; this is exactly what we observed with the H/A mutant (Figure 3c). To further probe this hypothesis, we have mutated this His residue to Lys. The resulting H/K mutant shows a membrane binding profile similar to that of cLac-wt (Figure 3c and 4), presumably because the Lys side chain can also interact with the PS carboxylate via a salt bridge. According to the docking model, the Asp and Gln residues appear to be buried at the protein-lipid interface, with Asp potentially forming a salt bridge with ammonium group of PS or PC. The buried positions of Asp and Gln demand a desolvation penalty for these residues upon lipid binding. Their mutation to Ala abolishes the desolvation penalty and makes the peptide more hydrophobic, which could be the underlying cause for the D/A and Q/A mutants to show compromised PS selectivity and bind PC-only vesicles (Figure 3c). Lending support to this desolvation penalty argument is the result of the Q, H/K mutant. Specifically, comparing Q, H/K to H/K, the Gln-to-Lys mutation does not improve the membrane binding propensity of the peptide, although Lys carries a positive charge and could potentially favor binding the anionic PS. The comparable membrane binding between H/K and Q, H/K is presumably because the larger desolvation penalty of Lys negates the benefit of charge-charge attraction between Lys and PS. This highlights the necessity to consider desolvation factors when one attempts to take advantage of charge-charge attractions to target lipid membranes (e.g., using cationic peptides to target anionic membranes). Collectively, the lipid binding profiles of the cLac variants can be rationalized

with the PS binding model of the parent protein lactadherin, indicating that the cLac peptide successfully mimics the parent protein for PS recognition.

Structural preorganization of cLac

It is challenging to fully characterize the structure(s) of membrane bound peptides in general given the heterogeneity and large size of model membranes. To gain preliminary insights into the structural aspect of the cLac variants, we measured the circular dichroism (CD) spectra of cLac-wt in presence and absence of PS-presenting model membranes. To avoid the interference of light scattering by lipid vesicles, we used a micelle-forming lysoPS (1-Oleoyl-2-Hydroxy-sn-Glycero-3-Phospho-L-Serine) for these CD experiments. Interestingly, similar CD profiles (Figure 6a) were observed for cLac-wt in PBS buffer alone and with 2 mM lysoPS, indicating cLac-wt in aqueous solution is conformationally quite well preorganized for membrane binding. In comparison to cLac-wt, all the mutants in PBS buffer showed varied degrees of loss of structure (Figure 6b). Specifically, the CD spectrum of cLac-wt displayed a global minimum at 223 nm and two minor features around 245 nm and 210 nm respectively. All the mutants lost the negative ellipticity at 245 nm. Similarly all cLac mutants displayed a reduction of ellipticity at 223 nm, with the D/A and Q/A mutants completely losing this global minimum. Although CD spectroscopy is generally considered a low resolution technique, the data presented here clearly demonstrate that cLac-wt is structurally preorganized for membrane binding, a feature well recognized for natural lipid binding proteins.¹² It is interesting to note that, despite the lack of structural preorganization, the D/A and Q/A mutants bind readily into vesicles with and without PS. This is presumably due to the increased hydrophobicity (no need of desolvation for the Asp or Gln residues) of these mutants, which dictates their partition into membranes.

Conclusion

This contribution documents our efforts to further understand the chemical basis of lipid recognition by peptides and proteins. Following our previous report on a PS-targeting peptide, we describe a new synthetic strategy for cyclic peptide synthesis, which takes advantage of the native chemical ligation (NCL) chemistry. This NCL based approach proves to be more efficient for cyclic peptide synthesis and labeling than the previously used protocol. A panel of cLac variants were successfully prepared and analyzed for their membrane binding properties. The results collectively support our conclusion that the cLac peptide effectively mimics the PS binding mechanism of lactadherin, in which multiple polar residues are conformationally preorganized by the protein or the cyclic peptide to balance various noncovalent forces (desolvation, hydrogen bonding and salt bridges) for specific PS recognition. These chemical insights should facilitate further development of synthetic peptides to target specific lipids.

Experimental Section

General Methods

Dawson (3-(Fmoc-amino)-4-aminobenzoyl) resin and HBTU were purchased from Novabiochem (San Diego, CA). 4-nitrophenylchloroformate, fluorescein-5-maleimide, Tris

base and Guanidine Hydrochloride were purchased from Thermo Fisher Scientific (Waltham, MA). 4-mercaptophenylacetic acid was purchased from Sigma Aldrich (St. Louis, MO). The phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Fmoc-(4, 4')-Biphenylalanine (Fmoc-Bip) was purchased from Chem-Impex Int'l Inc (Wood Dale, IL). All other Fmoc-protected and Boc-protected amino acids were purchased from Advanced Chemtech (Louisville, KY) or Chem-Impex Int'l Inc (Wood Dale, IL). Peptide synthesis was carried out on a Tribute peptide synthesizer (Protein Technologies, Tuscon, AZ). Peptide concentration measurements were performed on a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Peptide Synthesis and Characterization

The cLac peptides were synthesized through Fmoc/tBu chemistry using preloaded Dawson Dbz resin (3-(Fmoc-amino)-4-aminobenzoyl AM resin, Novabiochem), which consists of a 3,4-diaminobenzoic acid attached via the carboxyl group to the resin. The syntheses were carried out on a 0.05 mmole scale using five equivalents of the Fmoc-protected amino acids and HBTU for the coupling reaction. Additionally, a main-chain Boc-protected cysteine residue was installed at the terminal position of the peptide. A mixture of 50 mg 4-nitrophenylchloroformate in 1 mL of dichloromethane was added at the end of the synthesis and allowed to shake for 2 hours to afford the C-terminal imidazolidinone Nbz moiety, which will later facilitate the thioester formation in the subsequent native chemical ligation (NCL) reaction. The peptides were cleaved off the resin and globally deprotected using Reagent K (80% TFA, 5% H₂O, 2.5% EDT, 5% Thioanisole and 7.5% Phenol). The crude products were purified by RP-HPLC (Waters Prep LC, Juniper C18 Column). All peptides were characterized with LC-MS to confirm their identities and purities.

Native Chemical Ligation

The NCL reaction was carried by modifying a reported procedure.¹⁵ The linear peptide was dissolved in a mixture of DMF and ligation buffer (0.2 M sodium phosphate, 3 M guanidinium chloride, 20 mM 4-mercaptophenylacetic acid, pH 8) to a concentration of 2–5 mM. An equal amount of TCEP was added with respect to peptide concentration. The reaction was allowed to sit at room temperature with complete conversion in 4 hours. The reaction was acidified with water (0.1% TFA) and purified by RP-HPLC (Waters Prep LC, Juniper C18 column) to afford the cyclic peptide in 60–70% yield. All peptides were characterized with LC-MS analysis to confirm their identities and purities. The peptide concentrations were calibrated by measuring UV absorption at 275 nm ($\epsilon_{\text{Bip}, 275}=11200 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{Trp}, 275}=5378 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{peptide}, 275}=16578 \text{ M}^{-1} \text{ cm}^{-1}$).^{16,17}

Fluorophore Labeling

The labeling reaction was carried out by dissolving the cyclic peptide in DMF and adding one equivalent of fluorescein maleimide, 2% v.v N-methylmorpholine and DMF until the peptide concentration reached 200 μ M. Additionally, one equivalent of TCEP with respect to the peptide was added to ensure that the thiol was reduced and readily available for labeling. The reaction was left to sit at room temperature in the dark and complete conversion was achieved in 1 hour. The reaction mixture was purified by RP-HPLC (Waters Prep LC, Juniper C18 column) to afford the labeled product. All peptides were characterized with LC-

MS analysis to confirm their identities and purities. The peptide concentrations were calibrated by measuring UV absorption at 495 nm ($\epsilon_{\text{fluorescein}, 490}=80000 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{peptide}, 490}=80000 \text{ M}^{-1} \text{ cm}^{-1}$).¹⁸

Liposome Preparation

Dansyl labeled 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) was synthesized according to the reported protocol.¹⁹ Liposomes were prepared by dissolving and mixing the desired phospholipids in chloroform. The solvent was removed using high vacuum for 6 hours and the residue was suspended in 50 mM Tris buffer, pH 7.4. The lipid suspensions were treated through 20 cycles of a “freeze-and-thaw” process and extruded 21 times through a membrane with a diameter of 100 nm. The concentrations of liposome stocks were characterized via the Sterwart Assay.²⁰ Liposomes for the fluorescence anisotropy assay were prepared in the same manner as stated above, with the exception of incorporating the dansyl-labeled POPE.

FRET Binding Assay

The liposome binding of the cLac peptides were evaluated by monitoring the dansyl emission upon addition of peptides. The experiments were carried out on a SpectraMax M5 plate reader (Molecular Devices, Inc. Sunnyvale, CA) with a quartz cuvette of 10 mm path length. The samples were excited at 280 nm and their emission was monitored from 320 nm to 530 nm. All peptides were dissolved in DMF to give a 1 mM stock. The tests were done at a total lipid concentration of 100 μM and peptide concentration of 10 μM . The dansyl emission intensity at 520 nm was shown in Figure 3c for various lipid and peptide combinations.

Fluorescence Anisotropy Assay

The fluorescence anisotropy experiments were carried out on a Cary Eclipse Spectrophotometer (Agilent Technologies) with a quartz cuvette of 10 mm path length. Prior to measurements, a G factor of 1.4213 was calculated for the fluorophore on the instrument. All fluorescein labeled peptides were dissolved in DMF to give a 100 μM stock. The experiments were performed at a peptide concentration of 100 nM prior to liposome titration. The samples were excited at 495 nm and their emission was monitored at 520 nm to afford the anisotropy values. An increasing amount of liposome was titrated into the cuvette and the anisotropy values were measured at each concentration of liposome. The anisotropy values were plotted against liposome concentration to give the binding curves.

Circular Dichroism Measurements

The CD experiments were performed on an Aviv Model 420 CD Spectrometer (Aviv Biomedical, Inc., Lakewood, NJ). All samples for the wavelength scans were prepared in a buffer (PBS, pH 7.4) at a concentration of 50 μM using a quartz cuvette with a 2 mm path length. The CD spectra were recorded from 190 to 260 nm at 2 °C with a 10 second averaging time for each measurement. The lysoPS experiment was performed by titrating 2 mM of the micelles from a concentrated stock of 55 mM into the peptide sample and measuring as stated above.

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Figure 1.

Illustration of the peptide natural product cinnamycin, which is known to specifically recognize phosphoethanolamine PE. The cartoon representation of the lysoPE-cinnamycin complex was generated from the PDB file 2DDE, with the peptide showing its surface potential (blue: positive; red: negative) and lysoPE shown as sticks (C: green; O: red; N: blue; P: orange; H: white). The apparent K_d data were extracted from reference 5.

Figure 2.

Illustration of peptide cyclization strategies. a) On resin cyclization with side chain-immobilized Asp; b) peptide cyclization with native chemical ligation; c) exemplary HPLC traces of a cLac peptide before (left) and after (right) the cyclization reaction. 1 and 1*: unidentifiable impurities; 2: the linear precursor of cLac-wt; 2*: cLac-wt; 3: the Arg truncation product of 2 from solid phase peptide synthesis; 3*: the Arg truncation product of 2*; 4*: 4-mecaptophenylacetic acid. Both linear precursors (2 and 3) cyclized cleanly into the desired peptide macrocycles (2* and 3*).

Figure 3. Structure (a) and sequences (b) of the cLac variants and their binding propensities to vesicles of varied lipid composition (c). The results shown here were collected with peptides at 10 μ M concentration.

Figure 4. Membrane binding properties of the cLac mutants measured by fluorescence anisotropy of the fluorescein-labeled peptides. Vesicles containing 0% (PC only) and 20% PS were used to assess lipid selectivity of the peptides.

Figure 5.

A model of PS recognition by the protein lactadherin, which the cLac peptides were developed to mimic. The model was generated by docking a short chain (diacetyl) PS to the crystal structure of lactadherin.

Figure 6.

Circular dichroism analysis of the cLac variants. a) CD spectra of cLac-wt in PBS buffer with and without lysoPS micelles; b) CD spectra of the cLac mutants in comparison to cLac-wt. All samples were prepared with the peptide concentration at 50 μ M and the spectra were recorded in a cuvette with 2 mm light path.