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Cofactor-Free Detection of Phosphatidylserine with Cyclic Peptides Mimicking Lactadherin

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S Supporting Information

ABSTRACT: Cyclic peptides (cLacs) are designed to mimic the natural phosphatidylserine (PS) binding protein lactadherin. Unlike annexin V or its small molecule mimics, the cLac peptides selectively target PS-presenting membranes with no need for metal cofactors. We further show that a fluorophore-labeled cLac effectively stains early apoptotic cells. The small size and facile conjugation with a variety of imaging tracers make the cLac design promising for imaging cell death *in vitro* as well as in living organisms.

The lipid composition and distribution of cell membranes play an important role in regulating the physiology of the cell. For example, many signaling proteins can be recruited to the membrane by clusters of negatively charged lipids.[1−3] Not surprisingly, live mammalian cells actively maintain an asymmetric lipid distribution on their plasma membrane, with the most abundant anionic lipid phosphatidylserine (PS) completely confined to the cytosolic leaflet.[4] PS externalization has been observed for cells under abnormal development, including apoptotic cells,[5] activated platelets,[6] and virus-infected cells.[7] In fact, imaging PS exposure has been a benchmark approach to detect apoptotic cells in biological research. Considering most cancer therapeutics are designed to elicit apoptosis of cancer cells, noninvasive imaging of PS may enable facile evaluation of the efficacy of anticancer drugs.

Among the limited number of PS-targeting ligands,[8] annexin V has been most extensively investigated for detecting apoptotic cells *in vitro* and in living organisms. This 36-kDa protein, labeled with fluorochromes or radioactive nuclides, has been studied for imaging cell death in animal models of human cancer,[9] as well as in humans.[10] However, annexin V is less than ideal for PS imaging due to its large size, high cost, and calcium dependence of PS recognition. In addition, the moderate stability and slow clearance of annexin V severely limit its application in living organisms.[11,12] Small molecules that specifically target PS may circumvent the problems afflicting annexin V, and their development has captured increasing attention.[8] The elegant work from the Smith group resulted in a class of bivalent organozinc complexes that functionally mimic annexin V and preferentially associate with negatively charged membranes.[13−16] Recently, screening of phage-displayed peptide libraries has also yielded promising leads for PS recognition, although the molecular mechanisms of PS specificity have not been described for these peptides.[17,18] Herein, we report a new class of rationally designed small molecule receptors for PS. Taking a biomimetic approach, we grafted the key residues of the PS-targeting protein lactadherin into the scaffold of a cyclic peptide. In contrast to the organozinc complexes, the cyclic lactadherin mimics (cLacs) do not require any cofactors for PS recognition. We show that a fluorophore-labeled cLac selectively stains apoptotic cells by targeting PS on cell surfaces.

Lactadherin is a 47-kDa glycoprotein originally found in the milk secreted by mammary epithelial cells.[19] One of its key functions of lactadherin is to mediate the phagocytosis of apoptotic cells by recognizing surface-exposed PS.[20] Lactadherin stereoselectively binds to PS with a nanomolar affinity and without the need for cofactors.[21] Fluorescently labeled lactadherin is beginning to be used for PS detection on stored platelets[6] and cultured cancer cells.[22,23] The PS binding potential of lactadherin is localized to its C2 domain (Lact-C2),[24] the structure of which was recently solved through X-ray crystallography.[25,26] The lact-C2 structure displays a beta-barrel core and three loops projecting out for membrane insertion (Figure S1, Supporting Information (SI)). While the structure of the Lact-C2-PS complex remains elusive, the mutagenesis data as well as computational modeling suggest two potential binding sites (Surfaces C and D, as named by Shao et al.[26]) for the PS headgroup.[26] We focused on Surface D because computational docking experiments gave a more favorable binding free energy for the PS headgroup than PC (SI). Further structure examination revealed a number of residues critical for PS recognition (Figure 1): Phe81 and Trp26 belong to the group of hydrophobic residues that insert into the membrane; the hydrophilic residues (Asp80, His83, Gln95, and Arg148) engage in specific polar interactions with the PS headgroup that afford the lipid selectivity. The PS binding pocket looks like a bowl with the key residues displayed on the rim. Based on this observation, we hypothesize that the circular arrangement of these key residues can be recreated by the scaffold of a cyclic peptide.

Our first design (cLac-1, Figure 1) positions a Trp and a Phe next to each other to construct a membrane insertion motif. Three of the four polar residues (Asp80, His83, and Gln85) involved in PS binding are close in the Lact-C2's primary sequence. One exception is Arg148, which projects back into the vicinity of the other key residues in the folded protein. For the design of cLac-1, we linked Arg148 to the rest of the molecule by using two spacers: a dipeptide (-Gly-Gly-, Linker I) bridges Gln85 and Arg148, and a tripeptide (-Gly-Gly-Gly-, Linker II) links Arg148 and Trp26. Molecular modeling of cLac-1 shows that the two linkers will be sufficiently long and flexible to allow

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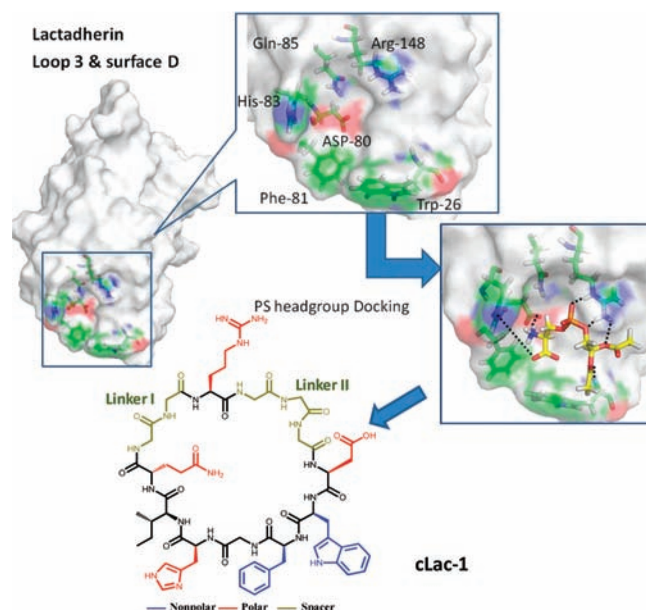

Figure 1. Structural modeling of Lact-C2 binding a short chain (diacetyl) PS, leading to the design of cLac-1 that mimics the PS binding pocket of lactadherin. The model is based on the Lact-C2 structure PDB: 3BN6.

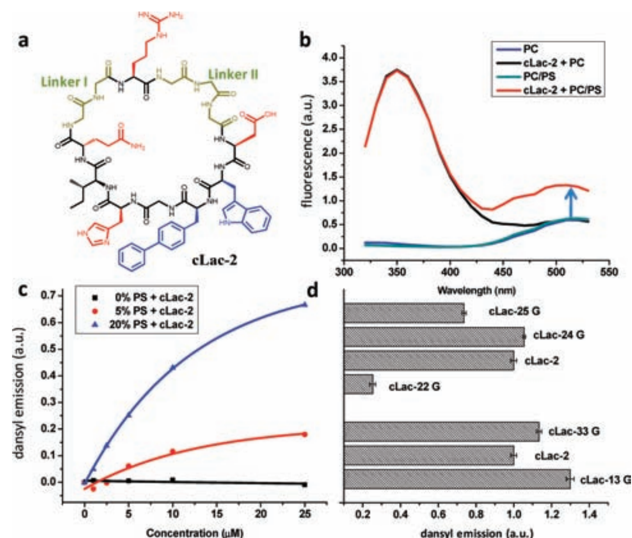

Figure 2. PS-targeting cyclic peptides. (a) Structure of cLac-2 that incorporates biphenylalanine (Bip) as a membrane anchor; (b) cLac-2 (at 25 μM) sensitizes dansyl emission (highlighted by the arrows) only with PC/PS (20%) liposomes; (c) concentration profiles of cLac-2 binding to membranes with varied composition; (d) SAR (structure−activity relationship) study of cLac-2 to compare the peptide variants with different linker lengths; all peptides are used at 25 μM concentration.

the native-like spatial arrangement of these residues for PS recognition.

The cyclic peptide was synthesized by using Fmoc/tBu chemistry (Scheme S1, SI). The main chain protected (with allyl) Asp was attached to Wang resin through its side chain carboxyl group. Standard solid phase peptide synthesis afforded the linear precursor with a deprotected N-terminus. Cyclization was accomplished on resin with HBTU-mediated amide bond formation after removal of the allyl protecting group on Asp. The peptide-membrane binding was evaluated by using a fluorescence assay: large unilamellar vesicles (~100 nm in diameter) were prepared with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholin (POPC) and varied percentages of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS). All vesicles contained 5% dansyl-labeled 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) to serve as a reporting group. The peptide-vesicle binding was monitored by the fluorescence resonance energy transfer (FRET) from Trp to the dansyl group. Our starting design cLac-1 displays no binding to lipid vesicles regardless of lipid composition (Figure S2, SI). Presumably, the peptide is too hydrophilic to partition into the membranes. This is perhaps not surprising given that additional hydrophobic residues (Leu28 and Phe31) are necessary for Lact-C2 to insert into membranes.[26]

A more hydrophobic variant of cLac (cLac-2, Figure 2a) was synthesized, which incorporates a biphenylalanine (Bip) to replace the Phe residue. The side chain of Bip has a cLogP value of 4.5, significantly greater than that of Phe (cLogP: 2.6). The FRET experiment shows no binding of cLac-2 to the PC-alone liposomes. However, mixing cLac-2 with the PC/PS vesicles clearly elicits a sensitized dansyl emission at 520 nm (Figure 2b). Furthermore, the sensitized dansyl emission becomes stronger with increasing concentrations of cLac-2. The titration results display a saturation profile, yielding an apparent dissociation constant (K_d) of ~8 μM. With cLac-2 at the saturating concentration (25 μM), the sensitized dansyl emission displays a linear dependence on the amount of PS presented by the membranes: vesicles containing 20% PS give close to 4-fold as much dansyl fluorescence as that of 5% PS (Figure 2c). It is worth noting that cLac-2 displays no net charge under experimental conditions, suggesting its selective association with PS-presenting membranes is not merely driven by the electrostatic attraction to the negatively charged surfaces. This compares favorably to the cationic peptide-based ligands[17] and the organozinc complexes,[15] which target PS predominantly through Coulombic interactions.

Further study of the structure−activity relationship shows that the linear precursor of cLac-2 (Lac-linear) binds to neither type of vesicle (Figure S3, SI), indicating that the circular arrangement of the key residues is necessary for the PS-dependent membrane association of the peptide. Our initial optimization of cLac-2 focused on the oligoglycine linkers. The number of Gly residues within Linker II was varied from 2 to 5. The Trp-to-dansyl FRET results (Figure 2d) show that cLac-22G (diglycine as linker II) elicits minimal dansyl emission, presumably because the linker is too short to allow the proper ring geometry for PS binding. Lengthening the linker to the tri- and tetraglycine moieties results in cLac-2 and cLac-24G respectively, which display a continued increase in dansyl emission, indicating an improved affinity of cLac for the membrane. Further extension to the pentaglycine linker affords cLac-25G that gave a reduced dansyl emission, presumably because the excessive flexibility of the linker causes an overwhelming entropic penalty toward membrane binding. These data indicate that the cLac-PS binding requires the cooperative action of multiple residues of the cLac peptide, analogous to the lipid binding mode of lactadherin, the protein that nature evolved for PS recognition. Interestingly, the peptide variants with varied lengths of Linker I (cLac-13G, cLac-2, and cLac-33G) exhibit a much less significant difference in the

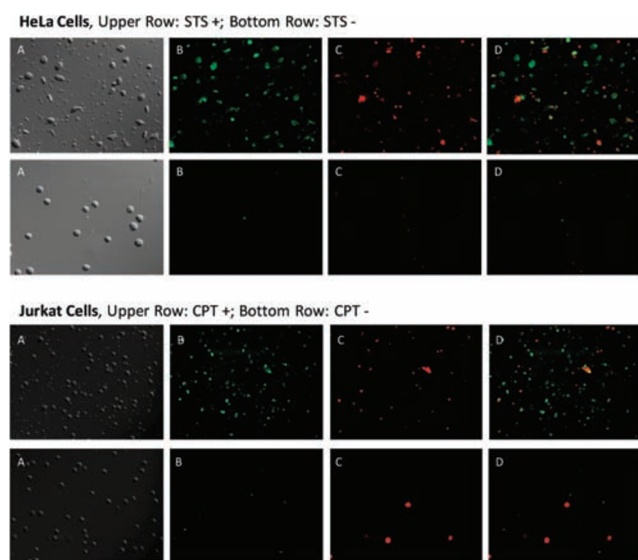

Figure 3. Microscopy analysis of cLac-2-Fl in staining apoptotic cells. (A) Phase contrast; (B) FITC channel; (C) PI channel; (D) overlay of B and C. Little staining of the nonapoptotic cells was observed for cLac-2-Fl.

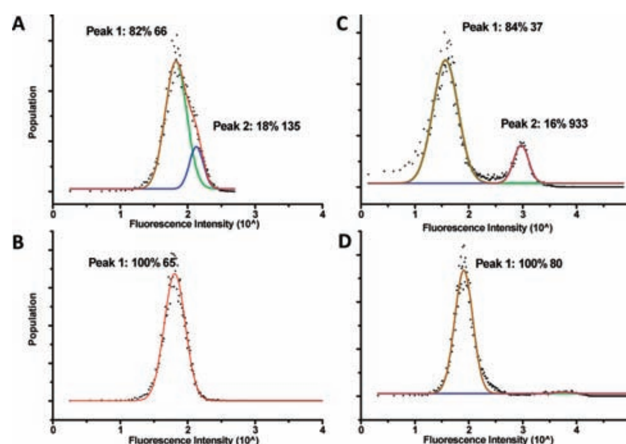

Figure 4. Flow cytometry results illustrating the effectiveness of the cLac peptide in labeling apoptotic cells. Jurkat cells treated with (A, C) and without (B, D) camptothecin were analyzed with cLac-2-Fl (A, B) and FITC labeled Annexin V (C, D) respectively. For each graph, the cell population is plotted against the fluorescence intensity (in log scale) of the cells. The percentage of cell population and the mean fluorescence intensity are labeled for each peak.

peptides' binding to membranes, possibly because the variation of Linker I can be effectively attenuated by the flexible Linker II (triglycine) on the other side of Arg148.

In order to evaluate the potential of cLac as an imaging reagent for apoptotic cells, we functionalized cLac-2 with a fluorescein label through the bioorthogonal click chemistry.[27,28] A propargyl glycine (Pra) was introduced to replace the Ile residue within the cLac-2 sequence. Based on the Lact-C2 structure (Figure S1, SI), the Ile side chain projects away from the protein–PS interface. Therefore we expect the Ile-to-Pra mutation to introduce minimal perturbation to the peptide–membrane association. Indeed, when the Pra derivative (cLac-2-Pra) was measured against liposomes with and without PS, membrane selectivity essentially identical to the parent peptide (Figure S4, SI) was observed. A fluorescein moiety was conjugated onto cLac-2-Pra to give cLac-2-Fl via the bioorthogonal click chemistry (SI), in which a flexible PEG linker bridges the fluorophore and the cyclic peptide. This design separates the fluorophore and the peptide so the specific binding of cLac and the membrane is minimally perturbed.

The fluorescent cLac was tested for its potential to stain apoptotic cells. Cultured HeLa cells were treated with the potent apoptogen staurosporine (STS) to trigger apoptosis. The experimental conditions were optimized with the FITC-labeled annexin V and propidium iodine (PI, a membrane impermeable DNA intercalator). In our hands, treatment with 1 μM STS for 3 h gave a large population of early apoptotic cells, which were stained with annexin V, but not PI (Figure S5, SI). Under these conditions, cLac-2-Fl (the green channel) readily stained STS treated cells, while little fluorescence was observed for the untreated cells (Figure 3). The lack of staining for the untreated cells indicates that cLac-Fl does not gain entry into healthy cells by diffusion or by endocytosis, as expected for most peptide-based agents. As a negative control, we tested the FITC-labeled Lac-linear, which gave little staining of the apoptotic cells under the same experimental conditions (Figure S6, SI). Collectively these results indicate cLac-2-Fl recognizes apoptotic cells through a specific mechanism. The merged microscopic image (channel D, Figure 3) exhibits minimal overlay between cLac staining and PI fluorescence, suggesting cLac largely detects early apoptotic cells. A similar phenomenon was observed for annexin V (Figure S5, SI). Since these early apoptotic cells hold their membrane integrity (negative for PI staining), we believe cLac2-Fl stained the cells by binding to their outer surfaces, presumably to PS.

To test the generality of the cLac peptide, we further examined its potential in imaging Jurkat cells with both microscopy and flow cytometry. Jurkat cells were treated with and without camptothecin (CPT) and then stained with cLac-2-Fl and the FITC labeled annexin V, respectively. Analogous to the HeLa cells, cLac-2-Fl readily differentiated the drug-treated versus untreated Jurkat cells (bottom panel, Figure 3). The cLac staining of Jurkat cells was quantitatively evaluated via flow cytometry analysis (Figure 4). Similar to annexin V, which stains ~16% of the CPT-treated cells, cLac-2-Fl labels ~18% of the cell population of the identical sample. Presumably cLac-2 and annexin stain the same subpopulation of the cells that is at the early stages of apoptosis. Lower fluorescence intensity was observed for the apoptotic cells treated with cLac-2-Fl than annexin V. This is partly because annexin V carries multiple copies of fluorophores (conjugated onto lysine side chains), while cLac-2 is labeled with a single dye. The relatively low PS-binding affinity of the cLac peptide may also contribute to the low fluorescence intensity observed for the apoptotic cell population.

In summary, we have developed a new class of cyclic peptides that successfully mimic the function of the natural PS-binding protein lactadherin. These cyclic lactadherin mimics (cLacs) selectively associate with PS-presenting membranes with low micromolar affinity. Furthermore, we demonstrate with two different cancer cell lines that a fluorescently labeled cLac effectively detects cells at the early stages of apoptosis. Importantly, the cLac-PS recognition does not require any metal cofactors and compares favorably to annexin V, whose PS-binding affinity is highly sensitive to calcium concentrations (see ref 29 and also Figure S8, SI). While the PS-binding affinity and the brightness of fluorescent labels remain to be optimized,

the cLac design benefits from its small size, ease of labeling, and cofactor-free PS recognition, which is highly promising for imaging apoptosis in cell cultures as well as in living organisms. In addition, the biomimetic approach that we used for cLac should be extendable to the development of small molecule receptors for other lipid molecules. Research toward these directions is currently ongoing.

ASSOCIATED CONTENT

Supporting Information. Supporting Information available on peptide synthesis and characterization, fluorescence labeling and imaging protocols, and results of the control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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