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Caged Mono- and Divalent Ligands for Light-Assisted Disruption of PDZ Domain-Mediated Interactions

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Supporting Information

ABSTRACT: We report a general method for light-assisted control of interactions of PDZ domain binding motifs with their cognate domains by the incorporation of a photolabile caging group onto the essential C-terminal carboxylate binding determinant of the motif. The strategy was implemented and validated for both simple monovalent and biomimetic divalent ligands, which have recently been established as powerful tools for acute perturbation of native PDZ domain-dependent interactions in live cells.

Understanding the complex dynamics of protein networks at the molecular level requires tools that allow for temporal and spatial control of specific protein−protein interactions (PPIs). The use of light as an effective switch to turn biological events on or off in live cells is a powerful approach because of its noninvasive nature and potential for high resolution. For instance, photolabile caging groups provide an efficient method for masking critical functionality in small bioactive molecules and protein-binding partners, preventing a functional interaction until the group is liberated through photoactivation.[1−3] This approach has been successfully applied to masking of neurotransmitters, nucleic acids, phosphopeptides, and proteins[2−5] and used to modulate specific PPIs by exploiting caged variants of molecules that compete with native interactions.[5,6] In this context, we were interested in developing a chemical caging approach for interactions mediated by PDZ (PSD-95/DLG/ZO-1) domains, which constitute an important and abundant PPI system in mammals.[7] Small globular PDZ domains are found in multidomain repeats or associated with other protein interaction domains and occur principally at cell junctions, where they are key players in the assembly and localization of macromolecular complexes involved in signal transduction pathways.[8−10] These types of interactions play a pivotal role in governing the dynamic localization of ion channels and receptors, particularly when they occur at synaptic junctions.[9] Therefore, they have been the focus of numerous studies involving the application of peptide-based ligands to acutely disrupt native interactions and define their functional roles in transient macromolecular complexes.[11,12] We recently introduced a series of synthetic biomimetic ligands that efficiently disrupt the synaptic PDZ domain-mediated interactions (PDMIs) involved in anchoring glutamate receptors (AMPAR[12a] and NMDAR[12b]) to macromolecular complexes in the postsynaptic density in live cells. While these ligands have provided valuable insights into PDMIs, the task of deciphering the various modes of regulation and their functional implications would greatly benefit from new tools that allow spatiotemporal control over these binding events.

Herein we report the design, synthesis, and characterization of caged PDZ domain ligands (PDLs) for the photochemical control and study of PDMIs. Indeed, this strategy should provide excellent spatiotemporal resolution of the acute disruption of endogenous PDZ domain binding events by controlling when and where a ligand can bind to its cognate protein partner. Our goals were both to develop a general caging method for PDLs and to implement the approach with recently reported biomimetic ligands.[12] To establish the general strategy and assess the caging efficiency, we exploited solvatochromic fluorophores for direct monitoring of the interaction of monovalent and bivalent peptide-based ligands and their cognate PDZ domains.[13,14]

As a first step in the design process, we obtained structural evidence that the fluorogenic probes described previously[14] constitute canonical PDLs, thereby providing a paradigm for this class of protein interactions. Crystallographic analysis of a PDZ domain bound to a prototypical fluorescent ligand established that the binding mode is comparable to that of other previously reported ligands,[8,15] thus excluding any perturbing role of the fluorophore and validating the use of the probes as representative PDLs (Figure 1 and Figure S1 in the Supporting Information). To design efficient caged ligands, we focused on conserved residues within canonical PDZ domain binding motifs to ensure both disruption of the critical interactions and broad applicability of this general strategy to different domain classes. Structural analyses[8,15] have consistently revealed that canonical binding of PDLs involves their C-terminal sequence, typically via the terminal amino acid hydrophobic side chain and carboxylate as well as the residue at the −2 position (Figures 1 and S2). The nature of the residue at the −2 position changes among various PDZ domains and is used to distinguish specific domains into classes.[16] Therefore, among these conserved interactions, the C-terminal carboxylate is the ideal target for caging because of its presence across all PDZ domain classes, essentiality for binding,

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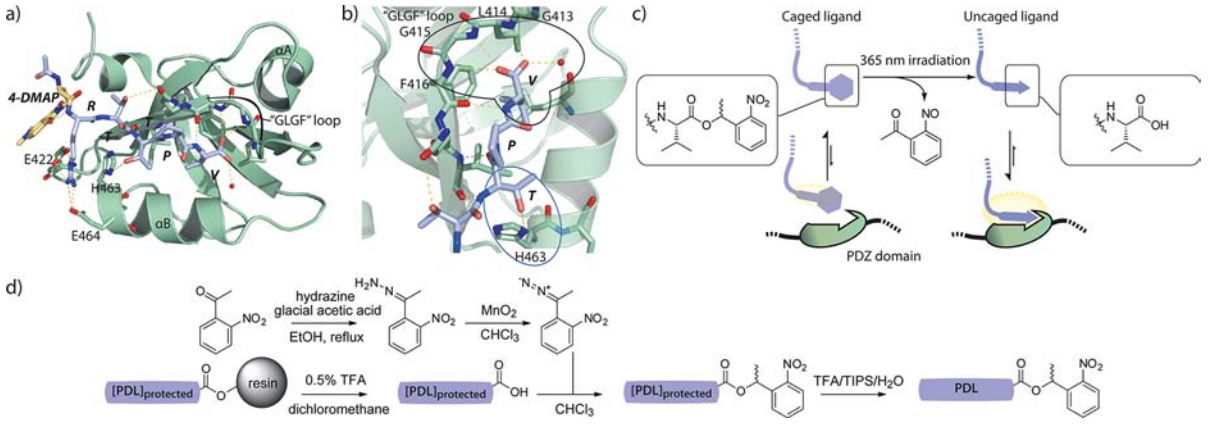

Figure 1. Caged PDL design and synthesis. (a) Structure of fluorescent probe **1** [stick representation in blue color theme, with the non-natural amino acid Dab(4-DMAP) in orange] bound to the third PDZ domain of SAP102 in green (PDB entry 3JXT). The hydrogen bonds between the ligand and the domain are highlighted in yellow. (b) Conserved PDZ domain−ligand interactions. In addition to less specific interactions of the PDL backbone, amino acids at position 0 (circled in black) and −2 (circled in blue) strongly interact with the domain surface residues in a conserved manner. (c) General caging strategy. Incorporation of a caging group such as NPE on the C-terminal carboxylate of a PDL should strongly impair its capacity to bind partner PDZ domains, which can then be recovered, when desired, by photorelease. Application of this strategy to PDZ domain fluorescent reporters allows direct monitoring of the interaction. (d) General synthesis of caged PDLs.

and intrinsic chemical reactivity. The introduction of a photolabile group at this position on a PDL should abolish key electrostatic interactions involved in binding to the domains and introduce steric hindrance, significantly impairing the interactions with PDZ domains (Figure 1).

The method used to obtain ligands caged at the C-terminal carboxylate is based on the introduction of a photolabile group after solid-phase peptide synthesis (SPPS). This strategy relies on activation of the caging group as a diazoalkane rather than the carboxylate as an activated ester to minimize potential epimerization of the terminal amino acid. The diazoalkane approach has been reported for caging of phosphate derivatives[17a] and amino acids[17b] and could be modified for use with peptide-based ligands. We chose the 1-(2-nitrophenyl)ethyl (NPE) caging group for its compatibility with biological applications[2] and facile chemical activation for reaction with the target carboxylates.[17] Peptides were first synthesized by standard Fmoc-based SPPS on a highly acid-sensitive resin (e.g., 4-carboxytrityl linker) and derivatized with the 4-dimethylaminophthalimide (4-DMAP) fluorophore[18] as reported previously.[19] The resulting peptides were cleaved from the resin with 0.5% trifluoroacetic acid (TFA), thus preserving the side-chain protecting groups to allow selective coupling of the free C-terminal carboxylate with a diazo-activated NPE group (Figures 1d and S3). The caging strategy was applied to the recently reported probes derived from the C-terminal sequences of Stargazin, GluN2A, and GluA1 that target representative class-I PDZ domains from synaptic PSD-95-like proteins (PSD-95, PSD-93, SAP97, and SAP102) and Shank3 (Table 1).[14]

The photolysis of the C-terminal caged peptides was first studied via HPLC by submitting a solution of caged ligand **3** to various durations of exposure to 365 nm UV light (Figure S4). Under these conditions, the amount of uncaged free acid **2** increased incrementally from 0 to >80% over 300 s while the amount caged of peptide decreased to 5%.

In parallel, the capacity of the caged and uncaged ligands to bind cognate PDZ domains was evaluated by monitoring the relative fluorescence increases in comparison with changes observed with noncaged probes (Figures 2 and S5). In the absence of protein, the caged and noncaged ligands showed

Table 1. Fluorescent Peptide-Based PDLs

Entry	Origin	Sequence[a]	Probe for[b]
1	Stargazin	Ac-φRTλPV-COOH	PLP-3
2	Stargazin	Ac-NTANφRTλPV-COOH	PLP-3
3	Stargazin	Ac-NTANφRTλPV-COONPE	n.a.
4	GluN2A	Ac-NRRVYKKλPφIESDV-COOH	PLP-12
5	GluN2A	Ac-NRRVYKKλPφIESDV-COONPE	n.a.
6	GluA1	Ac-NRRSGλPφGATGI-COOH	Shank-3
7	GluA1	Ac-NRRSGλPφGATGI-COONPE	n.a.

[a] φ = Dab(4-DMAP), where Dab = 2,4-diaminobutyric acid; λ = norleucine; Ac = acetyl group. The critical residues at positions 0 and −2 (blue) and the NPE caging group (red) are highlighted. [b] n.a.: not applicable. PLP-3 and PLP-12 stand for the third and the first two PDZ domains of *PSD-95-like proteins*, respectively.

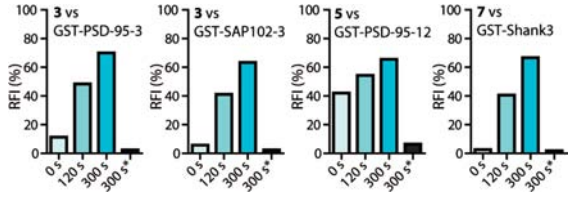

Figure 2. Generation of uncaged ligand by photolysis. Relative fluorescence increases (RFIs) for the caged probes in the presence of cognate PDZ domain(s) with increasing durations of 365 nm irradiation. RFIs were obtained by comparison with levels of fluorescence obtained with the same amount of the corresponding noncaged probes. The last entry in each graph (300 s*) shows the RFI obtained in the absence of PDZ domain(s) after irradiation for 300 s.

similar baseline fluorescence levels, indicating that the probe fluorescence was not affected by the presence of the NPE group in proximity to the reporter fluorophore (Figure S5). In the presence of the cognate PDZ domain, the caging group clearly resulted in significant loss of fluorescence, as the relative fluorescence increases were generally <12% of those of the noncaged ligand at $t = 0$ s (Figure 2), except for GST-PSD-95-12 (see Figure S5). Since we previously showed a correlation between fluorescence increase and affinity within a given series of ligands for the same PDZ domain, these observations reflect a lower binding affinity of the caged ligand.[14] Furthermore,

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exposure of the caged ligands to various durations of UV irradiation (t = 120 and 300 s) to release the caging group by photolysis and reveal the free C-terminal carboxylate resulted in the stepwise recovery of the fluorescence signal in the presence of PDZ domains. At t = 300 s, all of the ligands yielded fluorescence increases corresponding to more than ~70% of the maximum possible increase obtained with uncaged ligands, consistent with the uncaging levels observed by HPLC (Figure S4). UV irradiation at 365 nm did not result in significant photobleaching or other changes in fluorescence, thereby validating the use of fluorescence-based measurements to follow the modulation of PDMIs.

Next, the validity of the caging strategy was established by determining the relative binding affinities of the caged and uncaged peptides by fluorescence titration with their respective cognate domains (Table 2 and Figure S6). Binding saturation was not reached with the caged variants of the probes at concentrations well above those needed to achieve complete binding with free C-terminal carboxylate-containing ligands. The dissociation constants of the caged peptides were estimated to be at least 20-fold weaker than those of the parent peptides. We also observed that the fluorescence was much lower for all of the caged ligands, again confirming the weak affinities and the efficacy of the C-terminal carboxylate caging strategy.

Table 2. Dissociation Constants of Caged and Noncaged Probes for Their Respective Cognate PDZ Domains[a]

	noncaged		caged	
PDZ domain(s)	ligand	K_D (μM)[b]	ligand	K_D (μM)[b]
GST-PSD-95-3	**2**	1.66 ± 0.32	**3**	37.07 ± 4.95
GST-SAP102-3	**2**	5.45 ± 0.59	**3**	148.4 ± 82.8
GST-PSD-95-12	**4**	1.97 ± 0.26	**5**	82.9 ± 25.8
GST-Shank3	**6**	1.44 ± 0.30	7	59.9 ± 29.6

[a]See Figure S6 for the titration curves. [b]K_D ± standard error.

Having assessed the efficiency of C-terminal carboxylate caging in blocking PDZ domain−ligand interactions as well as the light-assisted recovery of the interactions upon photorelease of the caging group, we next probed the caged ligands for their capacity to compete against prototypical PDMIs upon exposure to light. For this purpose, we compared the effects of nonfluorescent caged and uncaged ligands at disrupting the interaction of a fluorescent probe with its cognate PDZ domain (**2** vs GST-PSD-95-3; Figure 3). Consistent with the observed loss of affinity after introduction of the caging group in the absence of 365 nm irradiation, the addition of a 20-fold excess of the caged nonfluorescent competing ligand **9** led to a minimal perturbation of the interaction, with a decrease of <7% in the fluorescence. In parallel, the same concentration of the noncaged version **8** reduced the fluorescence by >50%. However, after UV irradiation and uncaging of **9**, most of the competing properties of the peptide sequence were recovered, as judged by the significant loss of fluorescence comparable to that obtained with the noncaged ligand (40%). Application of the same treatment to the probe−PDZ domain pair did reveal a small amount of fluorophore photobleaching, albeit at levels that could not account for the effect observed after photoactivation of the caged competitor. This confirms the light-induced disruption of the probe−PDZ domain interactions.

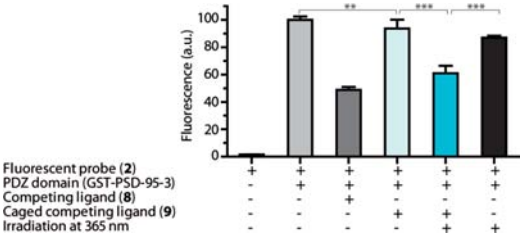

Figure 3. Light-assisted disruption of PDMIs. Competing properties of caged and noncaged ligands were estimated by observing the disruption of the binding of a fluorescent probe (5 μM) to its target PDZ domain (5 μM). Competing ligands were used in a 20-fold molar excess (t test: **, P > 0.1; ***, P < 0.02; $n \geq 3$).

We recently showed that a new class of biomimetic ligands comprising two covalently linked PDZ domain binding motifs acutely and specifically disrupt the synaptic stabilization of endogenous glutamate receptors in situations where monovalent ligands proved ineffective.[12] We were therefore motivated to implement the carboxyl caging strategy to develop efficient competitors of PDMIs in live cells. To take advantage of the validated bivalent biomimetic design while also maintaining optimal uncaging efficiency (i.e., requiring a single uncaging event for full recovery of the binding properties, as opposed to double uncaging if the divalent ligands were symmetrically caged), the original synthetic scheme of the ligand[12a] required a new strategy to allow for the introduction of a single caging group per divalent ligand (Figure 4a). After on-resin ligation, the original synthesis provided a symmetrical ligand dimer, which could not be easily used to introduce a caging group on only one of the C-terminal carboxylates. To generate the desired singly caged divalent ligand, we used copper-catalyzed azide−alkyne click chemistry to ligate the two forms of the ligand (caged and noncaged) in solution rather than on resin. The resulting monocaged divalent ligand derived from the Stargazin sequence was evaluated by a competition assay with the tandem PDZ domains of PSD-95 in comparison with both noncaged divalent and monovalent ligands (Figure 4b). The affinity constants confirmed that the insertion of a single caging group on a divalent ligand resulted in behavior comparable to that of the corresponding monovalent ligand (K_I = 0.49 ± 0.01, 14.2 ± 3.1, and 17.9 ± 0.7 μM for noncaged divalent **11**, monovalent **8**, and monocaged divalent **10**, respectively). In light of our previous studies,[12] it is evident that the caging and photorelease of the masking group provides a simple method to control the activity of these potent ligands for acute disruption of PDZ domain-mediated anchoring of the glutamate receptors at the synapse. These results also indicate that the general C-terminal

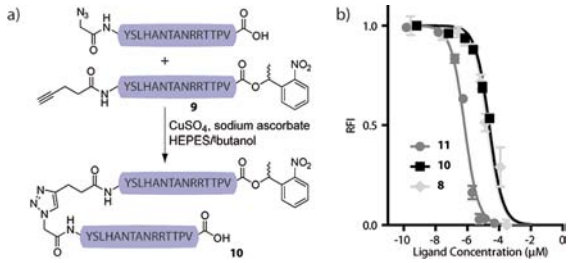

Figure 4. (a) Synthesis of monocaged divalent PDLs (see Figure S7 for details). (b) Fluorescence-based competition assay. The fluorescence resulting from the interaction of GST-PSD-95-12 and **2** was monitored as the concentration of nonfluorescent ligand (monovalent **8**, monocaged divalent **10**, or divalent **11**) was increased.

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caging approach as a method to control PDZ domain−ligand binding can be extended to more complex systems.

Finally, since the most important application of the tools presented here is to provide enhanced temporal control over the intracellular interactions mediated by proteins containing PDZ domains, we sought to estimate the stability of the ester bond connecting the ligand to the caging group in complex environments. Stability studies carried out using brain lysate preparations to approximate the conditions to which the caged probes would be exposed in cellular experiments provided clear evidence that the caged ligands, which are esters of peptides containing C-terminal valines, present a significantly higher resistance toward cellular esterases than do simple unhindered acetate esters (Figure S8). Specifically, after incubation for 20 min at 37 °C, the background hydrolysis of the caged ligand approached only ∼10%. In the context of our previous successful studies with noncaged ligands in cultured neurons,[12] this time window is sufficient to allow for efficient cellular internalization followed by uncaging experiments with minimal background hydrolysis. Moreover, the nature of the side chain of the C-terminal residue in most PDZ domain binding motifs (in this case valine) contributes significantly to the lower esterase susceptibility, thereby allowing for the generalization of our caging strategy. Finally, if additional resistance to cellular hydrolases is needed, the stability of the caged ligand could be further improved by a straightforward modification of the NPE caging group, as shown previously.[20]

In summary, we have described a general and versatile method to control the binding of a PDZ domain ligand to its cognate PDZ domains using photoactivation. The introduction of a caging group on the critical C-terminal carboxylate of a ligand-binding motif strongly impairs the interactions with targeted PDZ domains until a photorelease event is triggered. In view of the prevalence of C-terminal binding motifs for PDLs over less common internal sequences,[21] the approach should be generally applicable to most PDZ domain-mediated interactions. In a complementary approach, photoswitchable cyclic peptides for noncanonical internal binding motifs were recently reported.[22] Importantly, the caging method can be applied to more sophisticated bivalent ligands, and we have shown that a single caging group is sufficient to confer upon these ligands tailored binding properties that can be revealed by a single uncaging event. Such caged ligands will constitute unique tools for investigating systems where sharp spatial and temporal control of the release of the active ligand is critical, such as studies of the local effect of glutamate receptor trafficking on neighboring synapses and of the receptor life cycle with respect to diffusion or endo- or exocytosis. The new tools presented here provide high spatiotemporal resolution and can be used to study transient and localized biological events involving PDMIs.

■ ASSOCIATED CONTENT

Supporting Information

Supporting figures and experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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