

Sequential Activation and Deactivation of Protein Function Using Spectrally Differentiated Caged Phosphoamino Acids

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S Supporting Information

ABSTRACT: Photolabile caging groups, including the 1-(2-nitrophenyl)ethyl (NPE) group, have been applied to probe many biological processes, including protein phosphorylation. Although studies with NPE-caged phosphoamino acids have provided valuable information, these investigations have been limited to the use of only one caged species in a single experiment. To expand the scope of these tools, we have developed an approach for sequentially uncaging two different phosphopeptides in one system, enabling interrogation of multiple phosphorylation events. We present the synthesis of [7-(diethylamino)coumarin-4-yl]-methyl (DEACM)-caged phosphorylated serine, threonine, and tyrosine building blocks for Fmoc-based solid-phase peptide synthesis to allow convenient incorporation of these residues into peptides and proteins. Exposure of DEACM- and NPE-caged phosphopeptides to 420 nm light selectively releases the DEACM group without affecting the NPE-caged peptide. This then enables a subsequent irradiation event at 365 nm to remove the NPE group and liberate a second phosphopeptide. We demonstrate the versatility of this general sequential uncaging approach by applying it to control Wip1 phosphatase with two wavelengths of light.

Protein phosphorylation is a ubiquitous mechanism mediating protein function and dynamic signaling interactions. Regulated by a complex balance of kinases and phosphatases, phosphorylation governs fundamental physiological processes and can contribute to human disease.¹ Although traditional methods for interrogating the role of phosphorylation within the cell have provided valuable information,^{2,3} these approaches do not offer insight into the spatial and temporal dynamics of the modification, which are critical for proper cellular function. A complementary approach that overcomes this limitation is the use of photolabile protecting groups, or “caging groups”.⁴ These groups are covalently attached to an essential phosphorylated amino acid residue of a peptide or protein, masking the active component and rendering the molecule inactive within the biological system. Upon irradiation, the caging group is released, liberating the active phosphorylated species. The timing and spatial localization of uncaging are defined by the investigator, allowing precise control over the modification.⁵ We previously reported the synthesis of 1-(2-nitrophenyl)-ethyl (NPE)-caged phosphorylated amino acid building blocks for Fmoc-based solid-phase peptide synthesis (SPPS).^{6,7} While peptides

and proteins containing these residues have been successfully exploited for the investigation of many systems,^{8–12} the current caging approach is limited to the study of only one caged phosphopeptide or protein in an experiment. The development of orthogonal caging groups that can be independently released would significantly expand the scope of these tools by enabling interrogation of two phosphorylation pathways within one system using different wavelengths of light. Selective photolysis requires caging groups with distinct photophysical properties, and the few studies that have addressed this challenge have utilized caging groups from the *o*-nitrobenzyl (NB) and coumarinyl families.^{13–15} Kotzur et al.¹³ caged the sulfhydryl side chain of cysteine with derivatives from these two families to enable sequential deprotection in model peptide studies. Another report used NB-caged protein kinase G and coumarinyl-caged cAMP, which stimulates protein kinase A upon uncaging, to sequentially activate these protein kinase pathways.¹⁴ Finally, selective two-photon uncaging of dinitroindolyl-caged glutamate and coumarinyl-caged γ -aminobutyric acid was employed to modulate action potentials.¹⁵ All of these studies demonstrate the value of a sequential uncaging approach; however, they describe the synthesis of specialized molecules and do not provide methods that can be readily applied to other systems. To provide further advancement of the tools available to probe the role of phosphorylation *in vitro* and within cellular systems, we sought to develop a general sequential uncaging strategy that can be conveniently applied to diverse systems. Herein we detail the synthesis of coumarinyl-caged phosphorylated amino acid building blocks for Fmoc-based SPPS that can be used together with our previously reported NPE-caged phosphorylated residues to create peptides that can be sequentially uncaged. We then present the application of this system for photochemical control over the activity of wild-type p53 induced phosphatase (Wip1) (Figure 1).

Our previous work focused on the NPE group over other commonly used NB derivatives for application in a biological environment because photolysis at 365 nm is efficient and the nitrosoacetophenone photobyproduct is less reactive than the nitrosobenzaldehyde byproduct of NB groups.¹⁶ To establish a method for sequential uncaging of two different phosphopeptides in the same solution, we investigated the photolysis of the [7-(diethylamino)coumarin-4-yl]methyl (DEACM) caging group together with the NPE group. The DEACM group has been reported to be an effective cage for cyclic nucleotides¹⁷

Received: March 28, 2011

Published: June 21, 2011

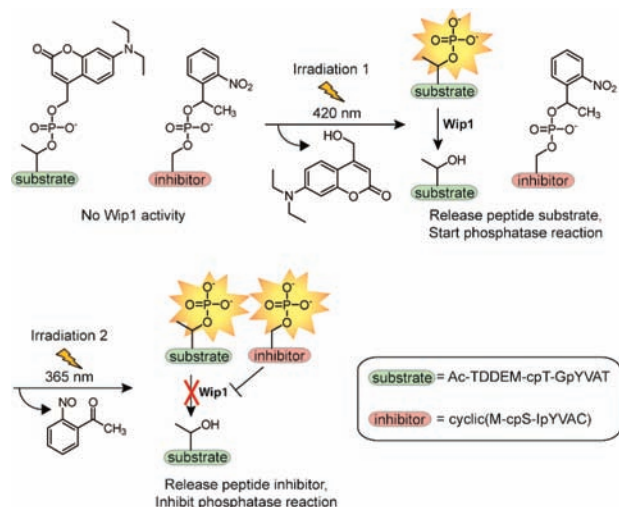


Figure 1. Sequential uncaging to control the activity of Wip1 phosphatase. The DEACM-caged phosphopeptide substrate enables the first irradiation at 420 nm to initiate the Wip1 reaction. A subsequent irradiation at 365 nm releases the NPE-caged phosphopeptide inhibitor to attenuate the Wip1 activity.

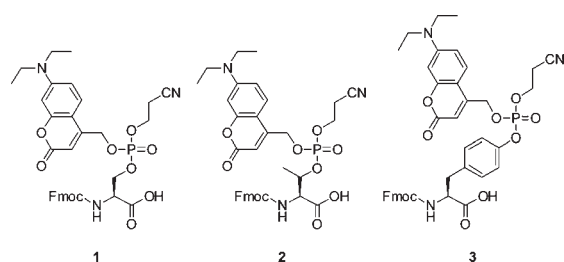
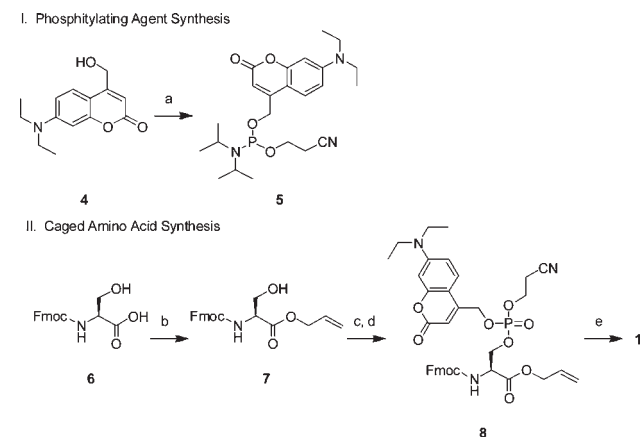


Figure 2. DEACM-caged phosphorylated serine, threonine, and tyrosine building blocks for SPPS.

and ATP,¹⁸ and uncaging of this derivative is very efficient.¹⁷ Importantly, the peak of maximal absorbance of the DEACM chromophore occurs at 390 nm,¹⁹ allowing it to be selectively released in the presence of the NPE group, which has minimal absorption at wavelengths above 380 nm (Figure S1 in the Supporting Information). Additionally, this coumarinyl group is advantageous in its own right because, relative to the NB family, the 7-diethylamino-4-(hydroxymethyl)coumarin (DEACM-OH) photobyproduct is inert in solution, and cellular systems that are sensitive to UV irradiation may benefit from long-wavelength activation.

As a model system, we chose Wip1 phosphatase, a Ser/Thr protein phosphatase central for attenuating the DNA damage and repair response and p53 tumor suppressor activity. It dephosphorylates and inactivates a number of proteins, including p38 MAP kinase and proteins that stimulate p53. Wip1 is involved in tumorigenesis, and the gene encoding it is amplified in some cancers.²⁰ A phosphopeptide substrate based on the phosphorylation site of p38 has been used for *in vitro* studies of Wip1 activity,²¹ and a cyclic phosphopeptide inhibitor of Wip1 has been identified.²² Thus, a DEACM-caged substrate and NPE-caged inhibitor constitute an ideal system to test the ability of the sequential uncaging scheme to initiate and then inhibit the

Scheme 1. Synthesis of DEACM-Caged Phosphoserine^a



^a Reagents and conditions: (a) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, triethylamine, CH₂Cl₂ (quantitative); (b) NaHCO₃/H₂O, Aliquat 336, allyl bromide, CH₂Cl₂ (91%); (c) 5, 4,5-dicyanoimidazole, THF; (d) *tert*-butyl hydroperoxide, 0 °C (75% over two steps); (e) Wilkinson's catalyst, 9:1 EtOH/H₂O (73%).

Wip1-catalyzed reaction in a wavelength-dependent and enzyme-independent fashion (Figure 1).

We synthesized DEACM-caged phosphorylated serine (1), threonine (2), and tyrosine (3) amino acid building blocks for Fmoc-based SPPS (Figure 2 and Scheme 1) to enable efficient incorporation of these caged residues into peptides and proteins. First, a DEACM-modified trivalent phosphitylating agent (5) was generated by reaction of DEACM-OH²³ (4) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite. Each commercially available Fmoc-amino acid was protected as the corresponding allyl ester, and the side-chain hydroxyl group of each derivative was treated with 5 to afford a DEACM phosphite intermediate. Subsequent *in situ* oxidation with *tert*-butyl hydroperoxide yielded the corresponding caged phosphoamino acid, and final allyl deprotection with Wilkinson's catalyst furnished the free-acid building blocks (1, 2, and 3) for peptide synthesis. Peptides were generated by standard SPPS employing PyBOP or HBTU/HOBt as coupling agents. The caged amino acid was coupled onto the amino terminus of the growing peptide using slightly modified conditions (HATU/HOAt and collidine) to prevent β -elimination of the phosphotriester, which can be a deleterious side reaction with serine and, to some extent, with threonine. After incorporation of the residue into the peptide, Fmoc removal with 20% 4-methylpiperidine in *N,N*-dimethylformamide concomitantly cleaved the β -cyanoethyl group to generate a mono-protected phosphopeptide that is resistant to β -elimination, thereby allowing peptide elongation to proceed under the standard conditions. The DEACM-caged peptides were routinely stored in the dark, but studies in which the caged peptides were maintained under ambient light revealed that the DEACM-caged residues are stable with half-lives ranging from 5–33 h, depending on the caged residue and peptide sequence (Table S1 in the Supporting Information).

We then synthesized NPE- and DEACM-caged phosphopeptides to explore selective release of the DEACM group in the presence of the NPE group (Table S2). The peptide sequences were based on a phosphopeptide substrate [Ac-TDDEM_pTG_pYVAT] and cyclic phosphopeptide inhibitor [cyclic(M_pS_pl_pYVAC)] of Wip1.

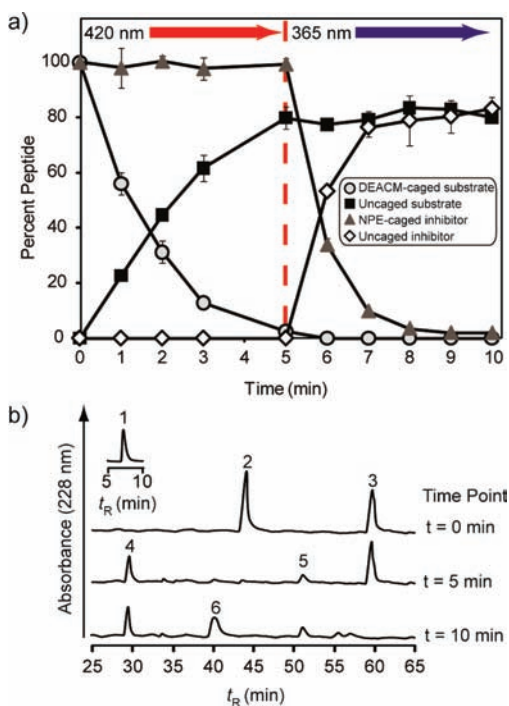


Figure 3. Time course of uncaging of DEACM-caged substrate and NPE-caged inhibitor peptides at 420 nm and then 365 nm. (a) A solution of the DEACM- and NPE-caged peptides ($113 \mu\text{M}$ each) in 10 mM HEPES (pH 7.1) with $70 \mu\text{M}$ inosine was irradiated at 420 nm and then at 365 nm. The reactions were followed by analytical RP-HPLC at 228 nm. Peptides were quantified on the basis of HPLC standard curves of the caged and noncaged derivatives. The amount of each peptide was normalized to the amount of inosine present, and the amounts of the uncaged peptides are plotted relative to the initial amounts of the corresponding caged species. (b) HPLC traces of the DEACM- and NPE-caged peptides (labeled as 2 and 3, respectively) before ($t = 0$ min) and after irradiation at 420 and 365 nm. The released substrate peptide, the DEACM-OH byproduct of uncaging, and the released inhibitor peptide are denoted as 4, 5, and 6, respectively. The inset shows the inosine peak (labeled as 1), which was used to normalize for the HPLC injection volume, on the same scale as the peptide traces.

Wip1 catalyzes the dephosphorylation of phosphothreonine (pThr) in the substrate peptide. Therefore, to prevent Wip1 activity until irradiation, the substrate peptide was prepared with DEACM-caged pThr. Additionally, because studies of Wip1 inhibition by cyclic phosphopeptides have revealed that phosphoserine (pSer) is necessary for inhibition, this peptide was synthesized with NPE-caged pSer to enable 365 nm light to drive inhibitor activation. Next, we used reversed-phase (RP) HPLC analysis to examine the kinetics of photolysis after exposure to irradiation from a hand-held light source equipped with a 420 nm cut-on filter (Figure 3). Under these conditions, irradiation of a mixture of the two peptides led to efficient release of the DEACM group and generation of the free pThr substrate peptide. Importantly, the NPE group was stable under these conditions, with only negligible uncaging even after irradiation for 3 min. Following this, irradiation at 365 nm released the NPE group to afford the corresponding pSer inhibitor peptide. These studies demonstrate that the two caging groups can be employed together to enable two uncaging events within one system. Because the DEACM group absorbs at 365 nm, it must be irradiated before the NPE group to achieve wavelength-selective photolysis.

Encouraged by the model uncaging studies, we tested the caged peptides in a Wip1 phosphatase assay, in which dephosphorylation of the substrate peptide was monitored by quantification of the released inorganic phosphate. Initially, we evaluated the caged peptides before and after irradiation to confirm that incorporation of the caging groups conferred the predicted properties. We compared Wip1 activity with the DEACM-caged substrate and the native, noncaged substrate (Figure S2). As anticipated, no activity was observed before irradiation, but exposure to 420 nm light for 3 min unmasked the pThr and restored the phosphatase activity to a level similar to that observed with the native substrate subjected to the same irradiation conditions ($0.52 \pm 0.03 \text{ s}^{-1}$ and $0.66 \pm 0.06 \text{ s}^{-1}$, respectively). On the basis of Michaelis–Menten kinetics with the reported K_M of the phosphopeptide substrate ($13 \mu\text{M}$),²¹ this activity corresponds to release of 53% of the phosphorylated substrate and is consistent with the time course of uncaging (Figure 3a).

We then turned our attention to the NPE-caged inhibitor peptide (Figure S3). To assess the effectiveness of caging pSer within the peptide, we added the caged inhibitor to an assay solution containing Wip1 and the DEACM-caged substrate after the reaction had first been initiated by irradiation at 420 nm. Without exposure of the caged inhibitor to 420 nm light, the activity was slightly reduced to $0.38 \pm 0.04 \text{ s}^{-1}$, demonstrating that masking the pSer in the peptide largely eliminates the inhibitory properties. Next, examination of Wip1 activity following irradiation of both the DEACM- and NPE-caged peptides at 420 nm was performed to ensure that this treatment did not significantly affect the NPE group. Indeed, irradiation of the two caged peptides at 420 nm for 3 min yielded Wip1 activity similar to that observed when the NPE-caged inhibitor was present but not exposed to 420 nm irradiation ($0.32 \pm 0.01 \text{ s}^{-1}$ and $0.38 \pm 0.04 \text{ s}^{-1}$, respectively). The modest inhibition resulted from minor release of the NPE-caged inhibitor under these conditions. On the basis of the model for competitive inhibition with the reported inhibitor K_i of $0.7 \mu\text{M}$,²² over 94% of the NPE-caged inhibitor was unaffected by the irradiation at 420 nm. This result is consistent with the time course of uncaging and demonstrates that photolysis of the DEACM group can occur independently of the NPE group. We then investigated the effect of liberating the NPE-caged inhibitor by irradiation at 365 nm. When the two caged peptides and Wip1 were irradiated together first at 420 nm and then at 365 nm immediately thereafter, the activity was significantly reduced to $0.10 \pm 0.02 \text{ s}^{-1}$ and was comparable to that resulting from the native noncaged inhibitor peptide (Figures S4 and S5).

The aforementioned sequential uncaging results are summarized in Figure 4, which shows the phosphatase activity before irradiation (region A) and after exposure to 420 nm light (region B) and then 365 nm light (region C). The Wip1 reaction was initiated upon irradiation at 420 nm and inhibited following exposure to 365 nm light. These experiments demonstrate that the system provides effective photocontrol over Wip1 activity by enabling two distinct biochemical processes to be activated in a wavelength-selective manner.

The sequential uncaging system can be applied to diverse systems to investigate the effects of multiple phosphorylation events within the same protein or within a signaling cascade. For instance, the scaffolding protein paxillin, which is essential for cell migration, is phosphorylated at multiple sites throughout the protein.²⁴ Semisynthesis of full-length paxillin¹² to incorporate two caged phosphorylated residues should enable interrogation of the influence of sequential phosphorylation at discrete sites.

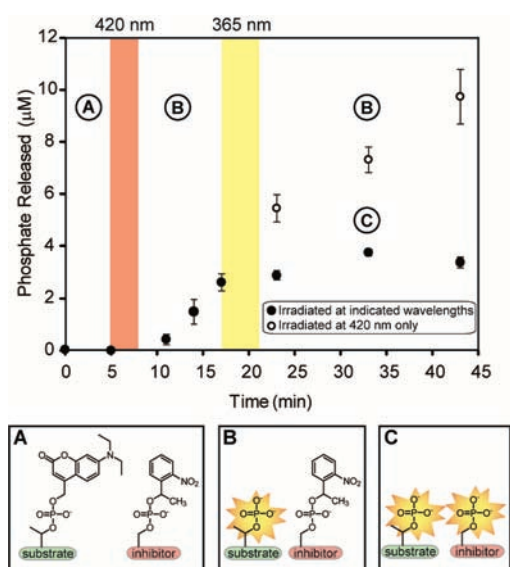


Figure 4. Wip1 phosphatase activity. Wip1 was incubated with the DEACM-caged substrate ($30 \mu\text{M}$) and the NPE-caged inhibitor ($5 \mu\text{M}$). No activity occurred before irradiation (region A), but exposure to 420 nm (orange bar) initiated Wip1 activity (region B). Subsequent irradiation at 365 nm (yellow bar) completely abolished the activity (region C). When the assay was irradiated only at 420 nm and not subjected to the 365 nm irradiation, the reaction continued.

Alternatively, as demonstrated through the Wip1 system, this sequential uncaging approach can be implemented to control processes that are regulated by phosphorylation events that exert opposing effects. In this regard, the sequential uncaging approach could be adapted to investigate the Rho family GTPase Rac, which can be activated and deactivated following phosphorylation of associated regulatory proteins.^{25,26}

While the studies with Wip1 have demonstrated the value of the sequential uncaging approach, this method could be further improved by decreasing the spectral overlap between the two caging groups. Although the DEACM group exhibits one of the most red-shifted absorption spectra among reported caging groups,^{17,19} the development of new derivatives that are activated at even higher wavelengths and that undergo efficient photolysis will increase the specificity of the first irradiation event when the NPE group is used in conjunction with these analogues.

In summary, we have developed a method for interrogating multiple sites of peptide or protein phosphorylation in one system by exploiting the distinct photophysical properties of the NPE and DEACM caging groups. Photolysis of the DEACM group at 420 nm efficiently releases it from the phosphopeptide while exerting a negligible effect over the NPE-caged peptide. A second irradiation event can then be used to liberate the NPE group. The DEACM-caged phosphoamino acid building blocks can be conveniently incorporated into any phosphopeptide or phosphoprotein that can be accessed by synthetic approaches, enabling investigations either in isolation or in concert with an NPE-caged molecule. This versatile method will facilitate detailed studies of previously inaccessible complex phosphorylation pathways.

■ ASSOCIATED CONTENT

S Supporting Information. Supporting figures, experimental methods, and NMR characterization of the caged amino acid

building blocks. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ACKNOWLEDGMENT

This research was supported by the NIH Cell Migration Consortium (GM064346). B.N.G. was supported by the NIGMS Biotechnology Training Grant (T32-GM08334), and A.A. was supported by a postdoctoral fellowship from the Swiss National Science Foundation.

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