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A Caged Phosphopeptide-Based Approach for Photochemical Activation of Kinases in Living Cells

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Kinase-mediated phosphorylation of proteins and lipids plays a central role in the regulation of intracellular signaling and cell functions in all eukaryotes.[1] Many kinases are maintained in an inactive state in quiescent cells and activated in response to cell stimulation, triggering the downstream pathways by phosphorylating their substrates. It is important that specific cellular decisions, such as cell-cycle checkpoints, differentiation, and cell migration, depend upon the precise temporal control and relative spatial distribution of activated proteins within cells. Analogous spatiotemporally defined, that is, cell- and stage-specific, activation of signaling pathways occurs during early development. Thus, the ability to artificially activate a kinase of interest at a desired time and/or location in cells as well as tissues or whole animals would provide important tools for investigating biological systems and ultimately for engineering living subjects.[2] Light-activatable, "caged" proteins are ideal for this purpose, as the activation can be triggered by a beam of light with high temporal and spatial resolution.[3] Along this line, several groups have previously reported photocaged derivatives of protein kinases[4] and kinase-regulated proteins,[5] and a few of them have found practical application in cells. Although protein caging is a straightforward approach to impart light sensitivity to proteins of interest, the strategy is limited by the difficulties associated with the preparation of semisynthetic caged proteins in vitro.

Herein we describe a new peptide-ligand-based method for photoactivation of kinases in living cells. Many intracellular kinases, which include Src family tyrosine kinases and phosphatidylinositol 3-kinase (PI3K), possess modular domains, Src homology 2 (SH2), and 3 (SH3) domains, that bind short peptide motifs containing consensus sequences.[6] The catalytic activity of these kinases is tightly regulated by the modular domain, and the peptide-binding induces the activation of the kinase in an allosteric manner.[7] We therefore reasoned that caged analogues of such modular domain-binding peptides should provide a general means to activate these kinases by light without need of chemical modification. To test this strategy, we targeted PI3K for photoactivation in this work. PI3K is a lipid kinase that catalyzes the phosphorylation of phosphatidylinositide-4,5-bisphosphate at the D3 position of the inositol ring, producing a lipid second messenger, phosphatidylinositide-3,4,5-trisphosphate ($PtdInsP_3$).[8] $PtdInsP_3$ activates a number of downstream signaling proteins and regulates diverse cellular functions, such as cell survival, metabolism, motility, vesicle trafficking, and neurite outgrowth. Although caged derivatives of various second messengers are currently available,[9] there is no strategy for photochemically producing $PtdInsP_3$ inside cells. Therefore, the ability to activate PI3K by light will be highly valuable for spatiotemporally controlling $PtdInsP_3$-dependent biological events in (multi)cellular contexts.

Previous studies have revealed that peptides containing a pYXXM (pY, phosphotyrosine; X, any amino acid) sequence can activate PI3K through binding to the SH2 domains within the p85 regulatory subunit.[10] Accordingly, we decided to prepare a SH2 domain-targeting peptide of which the activating tyrosyl phosphate moiety is protected with a photolabile group. Chemical synthesis of caged phosphopeptides have been recently described.[11,12] However, the peptides include classical 2-nitrobenzyl-based caging groups and thus the photosensitivity to less cell-toxic UV light (ideally over 350 nm) may not be sufficient for many applications. We thus first synthesized a new 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc)-caged phosphotyrosine building block **1** suitable for Fmoc-based solid-phase peptide synthesis (SPPS). Bhc is a recently developed caging agent which can be removed efficiently by both one-photon UV and two-photon IR excitation[13] and has been utilized for caging several biological molecules such as glutamic acid,[13a] mRNA,[13b] cyclic GMP/AMP,[13c] and protein-synthesis inhibitor.[13d]

The synthetic route to **1** is outlined in Scheme 1. In brief, the starting precursor 6-bromo-7-methoxymethoxycoumarin-4-ylmethanol **2** was prepared in three steps as previously reported.[13e] Phosphitylation of **2** with 2-cyanoethyl diisopropylchlorophosphoramidite and triethylamine (TEA) in dichloromethane (DCM) afforded the corresponding phosphoramidite **3** in quantitative yield. The *tert*-butyldimethylsilyl (TBDMS) group was used for transient carboxyl protection of commercially available *N*-α-Fmoc-L-tyrosine and the protected intermediate **4** was phosphytilated with **3** in the presence of tetrazole in tetrahydrofuran (THF). The phosphite was immediately

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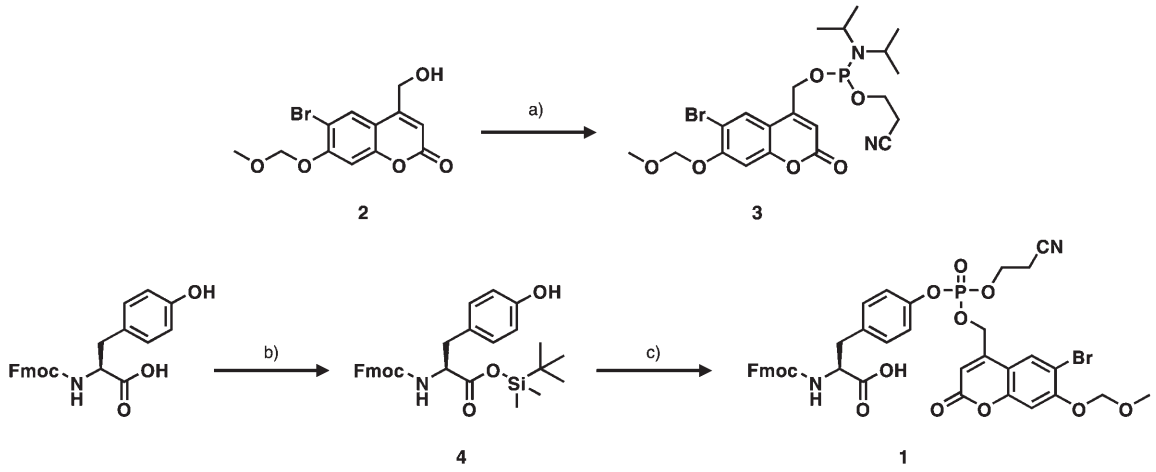

Scheme 1. Synthesis of Bhc-caged phosphotyrosine building block **1**. a) 2-cyanoethyl diisopropylchlorophosphoramidite and TEA in DCM. b) *tert*-butyldimethylchlorosilane and *N*-methylmorpholine in THF. c) i: compound **3** and 1*H*-tetrazole; ii: *tert*-butyl hydroperoxide (5–6 M solution in decane) in THF.

oxidized using *tert*-butyl hydroperoxide to the pentavalent species. Subsequent aqueous work-up gave the desired building block **1** in good yield. The cyanoethyl group on the phosphate is removed concurrently with Fmoc deprotection for chain elongation using 20% piperidine/*N*,*N*-dimethylformamide (DMF).[11] The methoxymethyl (MOM) group on the Bhc is cleaved at the final full deprotection step using TFA treatment. Using the building block **1**, a Bhc-caged phosphotyrosine peptide **5** was prepared by standard SPPS procedure and purified to homogeneity by reversed-phase HPLC (RP-HPLC; Figure 1A). **5** contains a sequence derived from 736–744 amino acids of platelet-derived growth factor receptor (PDGFR) in which the PI3K-activating pYXXM motif is included.[10]

The photochemical properties of the caged peptide **5** were evaluated. The peptide in K-MOPS solution was irradiated with UV light at 350 nm followed by RP-HPLC analysis as a function of time. Photolysis followed first-order kinetics and led to almost quantitative conversion of **5** into the corresponding phosphopeptide **6** and 6-bromo-7-hydroxycoumarin-4-ylmethanol **7**, which was confirmed by RP-HPLC and MALDI-TOF-MS analysis (Figure 1C). The quantum yield for photo-uncaging was determined to be 0.12, using the previously reported method (Figure 1D).[13] In addition, the overall photosensitivity, which can be expressed as the product of the quantum yield of photolysis (Φ) and the molar absorption coefficient (ε), was calculated to be 1538 $M^{-1}cm^{-1}$. The value is comparable with that for Bhc-caged cyclic GMP/AMP.[13c] We found that a 2-nitrophenylethyl-caged analogue of the same peptide (**8**, Figure 1B)[14] underwent photocleavage with an approximately eight times lower $\Phi\cdot\varepsilon$ value of 195 $M^{-1}cm^{-1}$ (Figure 1D), indicating the superior photosensitivity of the Bhc-caged phosphopeptide. On the other hand, **5** was stable to hydrolysis in the absence of light and less than 10% of the starting material was removed at room temperature even after one week. It should be noted that the marginal consumption of **5** in the dark was due to hydrolysis of tyrosyl phosphate ester, producing an unphosphorylated tyrosine peptide with no SH2-domain-binding ability. Furthermore, the two-photon uncaging action cross-section (δ_u) of **5** was measured by use of a femtosecond-pulsed, mode-locked Ti-sapphire laser. Product analysis by RP-HPLC after two-photon photolysis of **5** showed the same HPLC traces as those obtained after one-photon uncaging (data not shown), indicating that the Bhc-cage was efficiently liberated to afford **6** by IR laser pulses. The δ_u value determined at 740 nm excitation was 0.78 GM (Goeppert-Mayer, 1 GM = 10^{-50} cm^4s per photon), which ideally exceeds the 0.1 GM that is desirable for biological applications in live specimens.[13a] This represents a first report of two-photon-activatable caged polypeptides.

To demonstrate the photoactivation of PI3K in living cells, the green fluorescent protein (GFP)-fused Akt pleckstrin homology (PH) domain (GFP-PHAkt), a fluorescent indicator for $PtdInsP_3$,[15] was transiently expressed in NIH3T3 cells. Caged peptide **5** was microinjected into the cells and live-cell imaging was performed on a confocal laser scanning microscope (CLSM). Before light irradiation, GFP-PHAkt was evenly distributed throughout the cells (Figure 2). Exposure of the cells to the light of a UV hand lamp at 365 nm for 2 min and subsequent 15 min incubation led to the accumulation of the GFP-PHAkt at the plasma membrane, indicating in situ production of $PtdInsP_3$. A propidium iodide staining assay revealed that the UV irradiation caused no toxic effects on cells. When the same experiment was carried out after treating the cells with the PI3K inhibitor, LY294002 (LY), no production of $PtdInsP_3$ was observed. In addition, caged peptide **9**[16] containing a sequence specific to the SH2 domain of STAT3 (signal transducers and activators of transcription 3, Figure 1B) did not induce the accumulation of $PtdInsP_3$ even after light irradiation. All these results provide clear evidence that the photochemical uncaging of Bhc-caged phosphopeptide **5** induced the activation of endogeneous PI3K, producing $PtdInsP_3$ in living cells.

In summary, we have developed new caged phosphopeptides that are efficiently photolyzed in response to both one-photon UV and two-photon IR excitation. It will also be feasible to integrate Bhc-caged peptides with recombinant proteins by protein-ligation techniques[17] to yield multiphoton-activatable

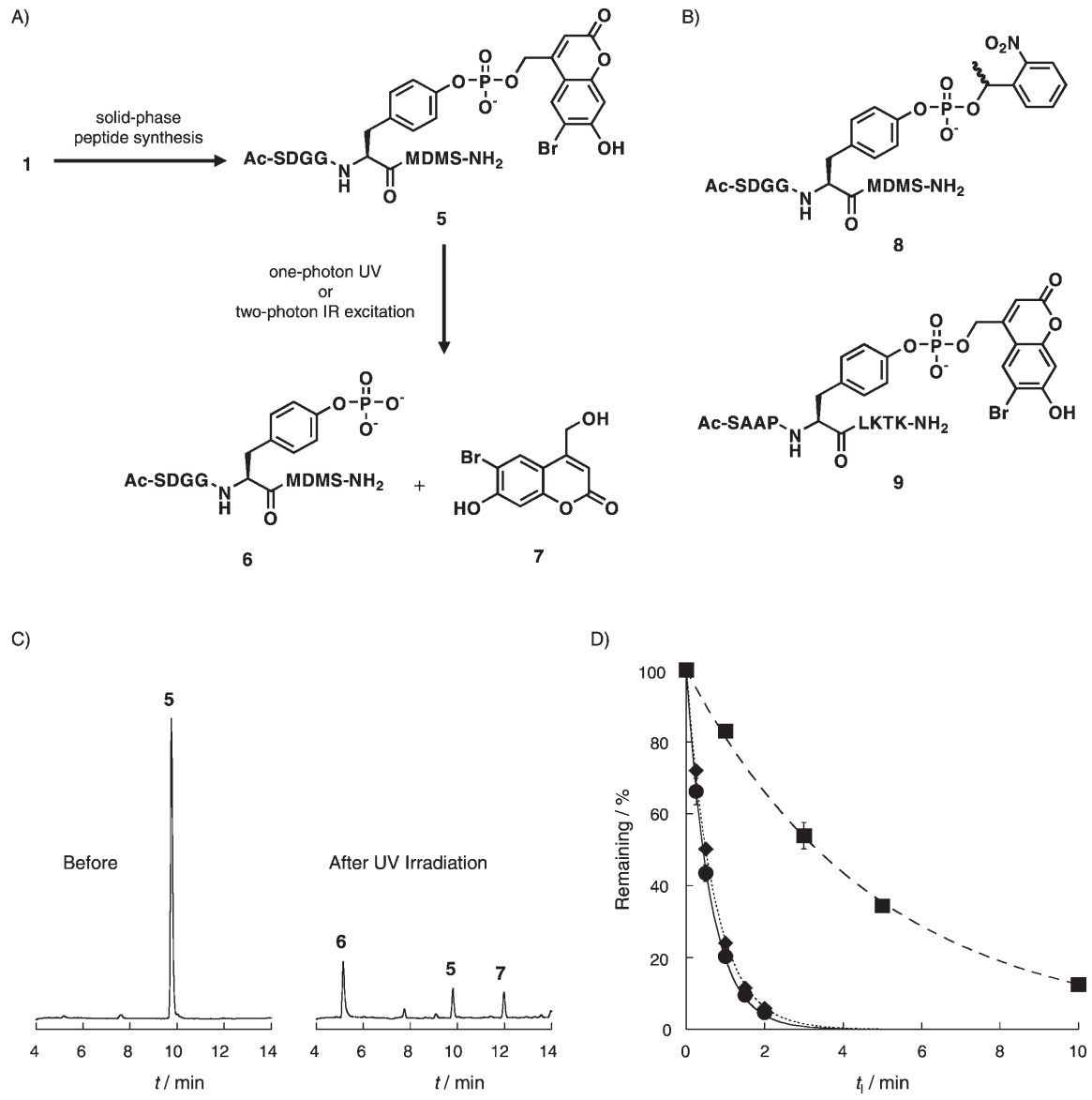

Figure 1. Synthesis and photochemical properties of Bhc-caged phosphotyrosine peptides. A) Building block **1** was incorporated into a PI3K-activating phosphotyrosine peptide **5** using standard solid-phase peptide synthesis. Caged peptide **5** releases the corresponding phosphopeptide **6** and Bhc-methanol **7** either by single-photon UV or two-photon IR excitation. B) Other caged peptides used in this study. C) HPLC traces before (left) and after (right) UV photolysis (350 nm, 4 min) of caged peptide **5**. Conditions: 100 μM in K-MOPS buffer. Detection at 220 nm. D) Time courses of photolysis of caged peptide **5** (●), **8** (■), and **9** (◆). Conditions: 8 μM in K-MOPS buffer.

semisynthetic proteins. We believe that the development and in vivo applications of multiphoton-activatable peptides/proteins are the next frontier in chemical biology. More importantly, we have demonstrated light-controlled PI3K activation and $PtdInsP_3$ production within living cells by using the caged phosphopeptide. We are particularly interested in applying the present tool to investigating the spatiotemporal role of $PtdInsP_3$ on cell migration[18] and neurite outgrowth.[19] Given the availability of several SH2 and SH3 domain-targeting polypeptides capable of activating kinases,[7,20] caged analogues of such peptide ligands should provide a general strategy for the activation of specific signaling pathways with temporal and spatial precision in the context of single cells, tissues, or whole organisms.

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Keywords: caged peptides · kinases · phospholipids · phosphopeptides · two-photon excitation

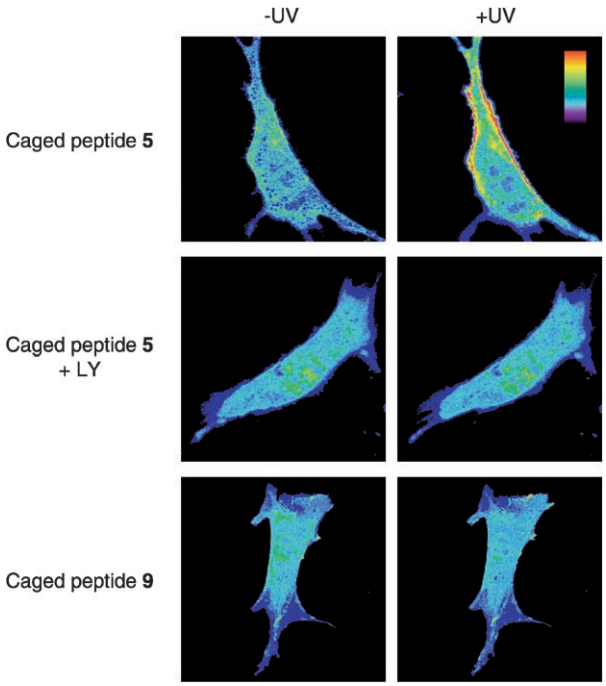

Figure 2. Light-mediated production of $PtdInsP_3$ in living NIH3T3 cells. NIH3T3 cells transiently expressing GFP-PHAkt were microinjected with caged peptide **5** or **9**. False-color images were acquired before and 15 min after UV irradiation (365 nm, 2 min) using a CLSM. In PI3K inhibition experiments, cells were treated with LY before microinjection.

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