Bioorganic Chemistry

Photocontrol of Smad2, a Multiphosphorylated Cell-Signaling Protein, through Caging of Activating Phosphoserines**

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The ability to activate proteins with spatial and temporal control inside live cells allows for quantitative kinetic measurements of protein function to be made in a biologically relevant context. Proteins that contain photolabile protecting groups appended to functionalities required for biological activity can be activated by light and provide a means to enable such analyses. Few reports of these reagents, known as caged proteins, have appeared in the literature because of difficulties in the preparation of such complex macromolecules.[1] Herein, we describe a semisynthetic route to the preparation of caged phosphoproteins. This strategy has been applied to the cellular signaling protein Smad2.

Smad2 is a key element of the intracellular response to cytokines of the transforming growth factor β (TGF-β) superfamily, which are involved in a myriad of normal and disease processes, including development, tissue homeostasis, and cancer.[2] Binding of TGF-β to its cognate receptor complex results in phosphorylation of the last two serine residues of the C-terminal sequence CSSMS of Smad2 (residues 463–467).[2] These phosphorylation events activate Smad2, which then disengages from the cytosolic retention factor SARA (Smad anchor for receptor activation), thus rendering the protein competent to both homotrimerize and interact with Smad4, a binding partner required for downstream functions.[2,3] Activated Smad2 accumulates in the nucleus, where it regulates transcriptional programs by interacting with a host of other proteins and target promoters.[2]

These differential protein–protein interactions and the localization of Smad2 provide a basis for understanding how this molecule functions in a cell. However, these descriptions are static and do not adequately describe the dynamics underlying these signaling events. Little is known about where many of these protein–protein interactions are initiated and for how long they exist. The kinetics of nuclear import and export as well as the importance of signal strength, duration, and localization are all poorly understood. We therefore targeted Smad2 for caging because the ability to activate this protein with temporal and spatial control allows one to directly address some of these fundamental issues.

Our caging strategy takes advantage of protein phosphorylation, the post-translational modification most often used to regulate protein activity.[4] Much recent effort has been directed at the preparation of caged analogues of phosphopeptides and phosphoproteins.[5,6] We used expressed protein ligation (EPL) as the center point of a semisynthetic scheme for the preparation of Smad2 whose activating phosphorylated residues were caged (Figure 1).[7] This approach offers several advantages, including the ability to produce caged proteins of any size in quantities sufficient for various biological applications without the need for mutagenesis.[7] Additionally, EPL readily allows for the installation of multiple caged phosphate groups in a homogenous manner.[7,8] This characteristic is of significant importance since many proteins, including Smad2, are controlled by multisite phosphorylation.[9]

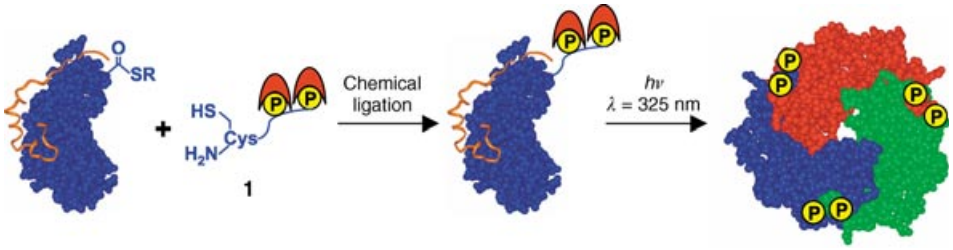

Figure 1. Semisynthesis of caged Smad2-MH2. Expressed protein ligation was used to ligate a recombinant Smad2-MH2-α-thioester/SARA-SBD protein complex to the doubly caged phosphopeptide **1** to give the caged Smad2-MH2/SARA-SBD heterodimer. Caged Smad2-MH2 is activated by exposure to UV light and subsequently releases SARA-SBD and forms a homotrimer. Smad2-MH2 is shown in globular form, SARA-SBD is shown in orange, phosphorylated residues are symbolized by yellow circles, and caging groups are symbolized by red crescents.

In this study we chose to work with the MH2 domain of Smad2 (residues 241–467, M_W = ca. 25 kDa), since its size enables precise characterization by chromatography and electrospray mass spectrometry (ESMS). The MH2 domain mediates many of the functions of Smad2, including receptor recognition, homo- and hetero-oligomerization, and nuclear import.[2] Full-length semisynthetic Smad2 has previously been prepared by EPL[3] and, therefore, we expect that the caging strategy outlined herein will translate effectively to the complete molecule.

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[**] We gratefully acknowledge Dr. Jean-Philippe Pellois, Dr. Jennifer Ottesen, and Edmund Schwartz for valuable discussions. We thank Claudio Alarcón and Prof. Joan Massagué for help with the nuclear import assay and Dr. Júlio César-Padovan and Prof. Brian Chait for MS/MS measurements. This work was supported by NIH grants CGM55843–07 and EB001991 (to T.W.M.). M.E.H. was supported by the NIH Medical Scientist Training Program grant GM07739.

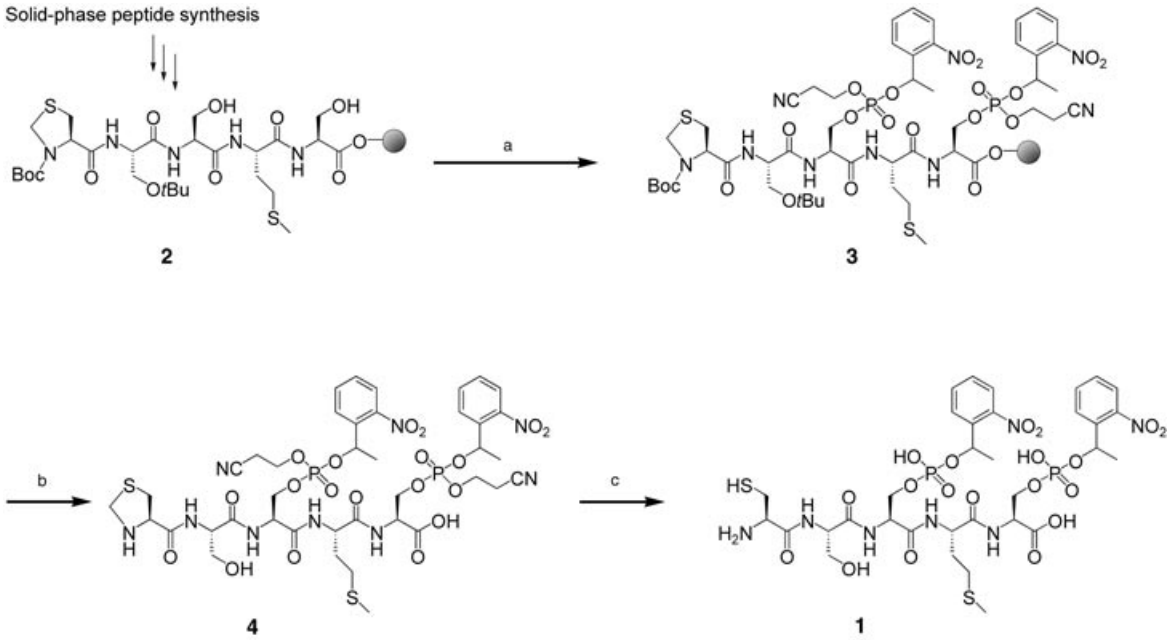
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/ange.200461141

Preparation of the caged phosphoprotein commenced with the synthesis of the corresponding doubly caged phosphopeptide **1** by using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy (Scheme 1). Key to the synthesis was 1) orthogonal trityl (Trt) protection of the side chains of the two serine residues to be phosphorylated and 2) incorporation of the N-terminal cysteine (Cys) group required for EPL as *tert*-butoxycarbonyl-1,3-thiazolidine-4-carboxylic acid (Boc-Thz). The latter allowed for the thiol group of Cys to be protected during the critical phosphorylation step and provided a convenient method for deprotection of the Cys residue following cleavage of the peptide from the solid support.[10] Following chain assembly and selective unmasking of the two serine residues, the resulting peptidyl resin **2** was dried extensively and treated with *O*-1-(2-nitrophenyl)ethyl-*O*′-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite,[5] which contains the 2-nitrophenylethyl (NPE) caging group. *tert*-Butylhydroperoxide was then used to oxidize the intermediate phosphites to the desired phosphates, thus yielding **3**. Notably, the undesired oxidation of the thioether of methionine to the sulfoxide was largely avoided (< 5 %) by limiting the oxidation time to 20 minutes (see the Supporting Information). Attempted on-resin removal of the β-cyanoethyl protecting groups from two juxtaposed phosphates resulted in significant amounts of β-elimination of the protected phosphate moiety.[11] Interestingly, this side reaction was not found to occur when the deprotection step was carried out in solution following cleavage from the resin. This observation suggests that the elimination was facilitated by the C-terminal ester linkage between the peptide and the solid support. Smooth removal of the β-cyanoethyl groups was therefore carried out under optimized conditions in solution using the hindered amidine 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Methoxylamine was then added in situ to convert Thz into Cys. The crude product contained one major compound (ca. 75 % by reversed-phase high-performance liquid chromatography (RP-HPLC), see the Supporting Information), which was subsequently purified to homogeneity to give the desired peptide that was caged on two phosphorylated serine residues (**1**) in 10 % yield.

The caged peptide **1** was labeled with fluorescein-5-maleimide (thus generating peptide **1**-**Fl**)[12] and subjected to low-intensity UV irradiation (312 nm, 2 mW cm^{-2}) followed by RP-HPLC to determine the kinetics and quantum yield of uncaging. Photolysis followed first-order kinetics with a rate constant of 4.9×10^{-3} s^{-1}, which corresponds to a quantum yield of uncaging of 0.16 per caging group (Figure 2). Interestingly, both possible singly caged peptides were observed in approximately equal amounts after irradiation for an intermediate length of time, thus indicating that the efficiency of photolysis was equivalent for both caging groups (see the Supporting Information). Brief (< 5 s) exposure of the peptide to the output of a He-Cd laser (325 nm, 4.74 W cm^{-2}) resulted in near quantitative conversion (> 97 %) into the uncaged peptide (see the Supporting Information).

A recombinantly expressed Smad2-MH2 domain (residues 241–462) bearing a C-terminal thioester was prepared as previously described.[3] A complex of this protein with the minimal Smad binding domain of SARA (SARA-SBD, residues 665–721) was formed by incubation with excess SARA-SBD and purified by cation-exchange chromatography. The resulting pure protein complex was concentrated to 0.25 mM and a fourfold molar excess of the caged peptide **1** was added to initiate the ligation reaction (Figure 1). The reaction was monitored by RP-HPLC, ESMS, and sodium



Scheme 1. Synthesis of doubly caged phosphopeptide **1**. a) 1. *O*-1-(2-nitrophenyl)ethyl-*O*′-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite, 4,5-dicyanoimidazole, DMF (anhydrous); 2. 1 M *t*BuOOH, CH_2Cl_2 (anhydrous); b) 92.5 % TFA, 2.5 % EDT, 2.5 % TIS, 2.5 % H_2O; c) 1 % DBU, DMF then 0.5 M $MeONH_2$·HCl, H_2O. TFA = trifluoroacetic acid, EDT = 1,2-ethanedithiol, TIS = triisopropylsilane.

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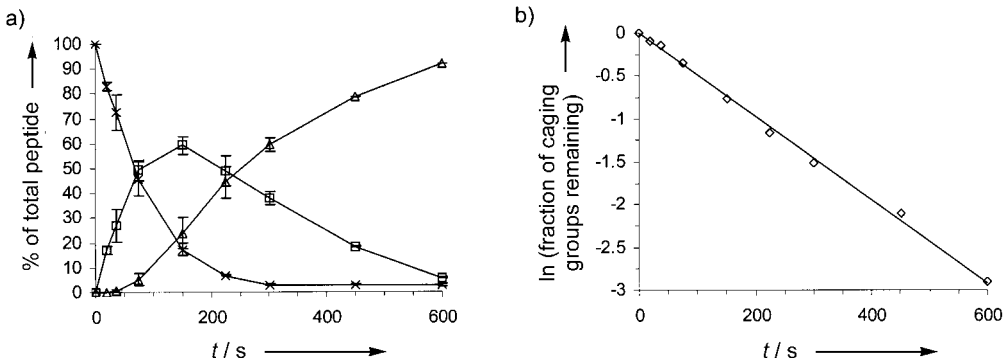

Figure 2. Photolysis kinetics of doubly caged phosphopeptide **1** labeled with flourescein-5-maleimide (**1-Fl**). a) A solution of **1-Fl** at 10 μM was irradiated with low-intensity UV light (312 nm, 2 mW cm^{-2}) for the times indicated and subjected to RP-HPLC for quantitation of the doubly caged (×), singly caged (□), and uncaged forms (△). The percentage of each is plotted versus time of irradiation. b) The fraction of caging groups remaining was calculated and the natural logarithm at each time point plotted versus time of irradiation, along with a line of best fit, which yielded a first-order rate constant for photolysis of 4.9×10^{-3} s^{-1} and an r^2 value of 0.99. The mean of two experiments is plotted for both (a) and (b), and the standard deviation is represented by error bars in (a).

dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and was complete after 12 h. The caged protein was purified by preparative size-exclusion chromatography (SEC) and its identity was confirmed by ESMS (Figure 3c).

To be deemed effective, the caged protein should behave as if it was nonphosphorylated in the absence of UV light and should display all the properties of the active, doubly phosphorylated Smad2-MH2 when uncaged by UV light. We therefore proceeded with studies designed to determine the oligomerization state of the caged protein before and after UV irradiation. Gratifyingly, the caged protein mimicked nonphosphorylated Smad2-MH2, since it bound SARA-SBD in a 1:1 molar ratio (Figure 3a). This heterodimeric arrangement of Smad2-MH2 and SARA-SBD was verified by SEC coupled with multi-angle laser light scattering (MALLS) detection at a loading concentration of 5 μM (see the Supporting Information).[13] MALLS analysis indicated that the caged protein had a slight residual tendency to form homotrimers at higher loading concentrations (25–50 μM; see the Supporting Information). Indeed, in preliminary studies where only one caging group was installed on either phosphoserine 465 or 467, this tendency to oligomerize was even more pronounced (data not shown). Importantly, the tendency of the doubly caged protein to homo-oligomerize is concentration-dependent, such that at physiologically relevant concentrations (< 5 μM)[14] this behavior is no longer observed. Brief irradiation (< 5 s) of the caged protein with the output of the He-Cd laser followed by SEC, RP-HPLC, and ESMS demonstrated that the caging groups were quantitatively removed from the protein and that SARA-SBD was released from Smad2-MH2 in favor of homotrimerization (Figure 3).

As a step toward our ultimate goal of using caged phosphoproteins in live cells to study the kinetics of biological

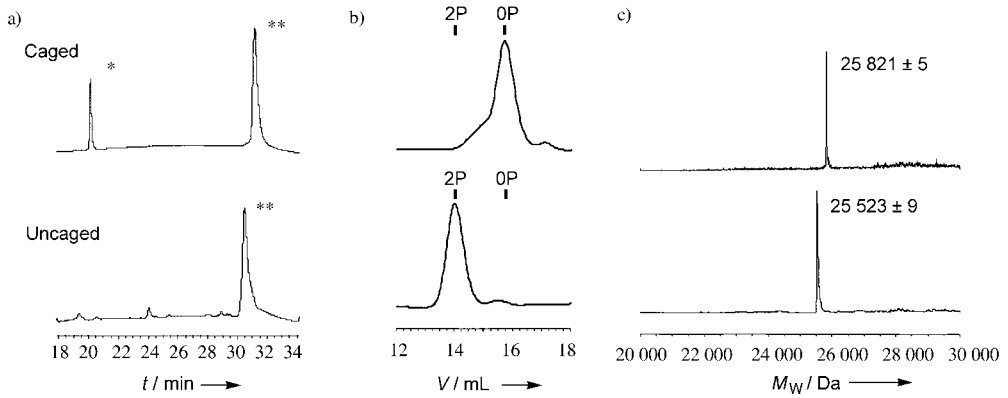

Figure 3. Characterization of caged (top panels) and uncaged (bottom panels) Smad2-MH2. Caged Smad2-MH2 was converted into uncaged Smad2-MH2 by irradiation for 5 s with a He-Cd laser (325 nm, 4.74 W cm^{-2}). a) The ratio of caged and uncaged Smad2-MH2 to SARA-SBD was determined by subjecting the caged and uncaged proteins to RP-HPLC with detection at 214 nm and integrating the peaks corresponding to SARA-SBD (*) and Smad2-MH2 (**). The peak area ratio of caged Smad-MH2:SARA-SBD is 4:1, which at 214 nm indicates a 1:1 molar ratio. b) The homo-oligomeric status of caged and uncaged Smad2-MH2 was assessed by SEC with detection at 280 nm. The elution positions of doubly phosphorylated Smad2-MH2 (2P) and nonphosphorylated Smad2-MH2 (0P) controls are indicated. c) The reconstructed molecular weight from ESMS indicates that the caged protein (calcd $M_W = 25\,818$ Da) was assembled successfully. ESMS of the uncaged protein (calcd $M_W = 25\,519$ Da) indicates quantitative removal of the caging groups after laser irradiation. Observed molecular weights are also shown.

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signaling and transport processes, we set out to determine the behavior of caged Smad2-MH2 in a nuclear import assay. When incubated with digitonin-permeabilized HeLa cells in the presence of SARA-SBD, nonphosphorylated Smad2-MH2 (OP) is excluded from the nucleus, whereas phosphorylated Smad2-MH2 (2P) accumulates in the nucleus (Figure 4).[14] UV irradiation had no effect on the localization

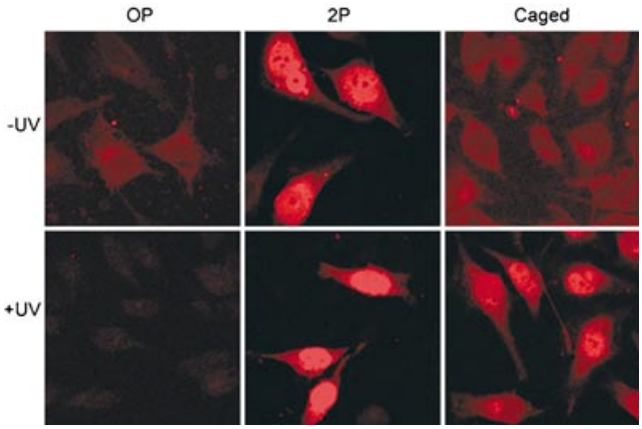

Figure 4. Nuclear import assay of Smad2-MH2 variants labeled with Texas Red C_2–maleimide. Nonphosphorylated (OP), doubly phosphorylated (2P), and caged Smad2-MH2 before (top panels) and after UV (bottom panels) laser irradiation (as in Figure 3) were incubated separately at 1.5 μM with digitonin-permeabilized HeLa cells for 20 minutes at room temperature in the presence of 4.5 μM GST-SARA-SBD (GST = glutathione S-transferase), an ATP-regenerating system, and 1 mg mL^{-1} bovine serum albumen (BSA). After the import reaction, cells were washed, fixed, and analyzed by confocal microscopy for the localization of each Smad2-MH2 variant. A control experiment was performed in which the import reactions were carried out on ice. This treatment prevented nuclear accumulation (data not shown), which is consistent with nuclear import of Smad2 being an energy dependent process, as previously demonstrated.[14]

pattern of phosphorylated and nonphosphorylated Smad2-MH2 control proteins (Figure 4). In the same assay we found that caged Smad2-MH2 was excluded from the nucleus, whereas uncaging of the protein with UV light led to dramatic nuclear accumulation (Figure 4). This demonstrates that the caged and uncaged proteins behave as desired in a biological context.

In summary, we have prepared Smad2-MH2 caged on two activating phosphate residues by a semisynthetic route. The molecule described represents the first report of a protein caged on a phosphate group. In principle, this approach can be applied to the construction of a caged version of any protein activated by phosphorylation. We are currently investigating this and other strategies of molecular photocontrol over protein function with live cell-imaging techniques.[15] These studies are expected to yield quantitative insight into the kinetics of Smad2 nuclear import and export. Additionally, we plan to address fundamental questions regarding the importance of signal strength and duration in biological processes.

Received: July 1, 2004

Keywords: bioorganic chemistry · phosphorylation · photochemistry · proteins · semisynthesis

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