Communication

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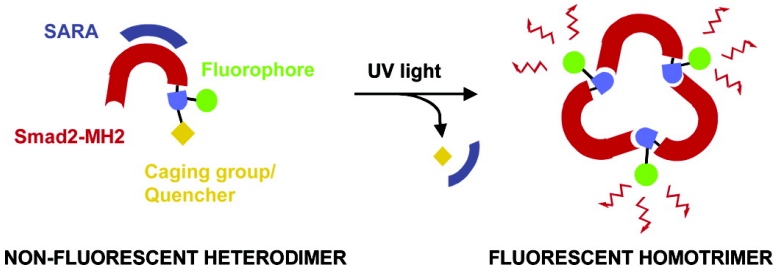
J. Am. Chem. Soc., **2004**, 126 (23), 7170-7171• DOI: 10.1021/ja0499142 • Publication Date (Web): 21 May 2004



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Published on Web 05/21/2004

Simultaneous Triggering of Protein Activity and Fluorescence

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The characterization of molecular processes as they occur inside cells is essential to a comprehensive understanding of living organisms. Although several genetic and chemical approaches have been employed successfully to undertake such a challenge, there is still a need for the development of new methodologies.[1] For example, to determine the exact site, timing, and kinetics of cellular events, ideal tools would provide for real-time monitoring of the location of a specific protein, spatial and temporal control of its activity, and facile differentiation between its active and inactive state. We reasoned that if protein photocaging[2] and fluorescence labeling were combined, one might achieve these desirable features. Our approach consists of modifying a fluorescently labeled protein with a photocleavable group that can both suppress the protein activity and quench its fluorescence. Photocleavage of this caging group results in the simultaneous restoration of protein activity and luminescence of the fluorescent tag. This strategy was applied to Smad2, a key protein involved in the transforming growth factor β (TGF-β) signaling pathway.

TGF-β signaling is involved in various cellular processes, including cell proliferation, differentiation, and apoptosis.[3] Signaling from the cell membrane to the nucleus is mediated by Ser/Thr kinases and the Smad family of proteins. Specifically, the binding of a particular ligand to transmembrane receptors leads to the phosphorylation of Smad2, a receptor-regulated Smad, on the last two serine residues of the C-terminal sequence CSSMS (residues 463−467). Phosphorylation results in the release of Smad2 from the membrane-anchored protein SARA, binding to the co-mediator protein Smad4, translocation into the nucleus, and regulation of target gene expression. The crystal structure of a phosphorylated Smad2, along with biochemical characterization of the protein prepared by semi-synthesis, revealed a homotrimeric arrangement directly mediated by the phosphoserines.[4] Structural analysis of the homotrimer showed that the C-terminal carboxylate contributes significantly to the stabilization of the complex by making several hydrogen bonds with neighboring residues. Covalent attachment of a caging moiety at this site could therefore disrupt these interactions and maintain the phosphorylated protein in an inactive SARA-bound state, while irradiation with UV light and subsequent cleavage of the caging group would unmask the active trimeric Smad2. A photochemical trigger that mimics the critical biochemical event of serine phosphorylation involved in the signaling pathway would therefore be obtained. Furthermore, the protein structure also suggests that the side chain of Met466 is not involved in any interactions, therefore offering a convenient site for the introduction of a noninvasive fluorescent tag.

Expressed protein ligation (EPL)[5] was used to synthesize a protein that contains two phosphates, a fluorescent probe, a fluorescence quenching molecule, and a photocleavable linker (Figure 1). The linker 4-[4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy]butanoic acid (photolinker, pl) was chosen as a bifunctional caging group.[6] Preliminary analysis on model peptides demonstrated that fluorescein (Fl) and dabcyl (Dab) were a suitable pair for efficient fluorescence quenching (data not shown). The peptides H-CSSMS-OH (**1**), H-CSpSMpS-OH (**2**), H-CSpSK(Fl)pS-OH (**3**), H-CSpSMpS-pl-K-NH_2 (**4**), and H-CSpSK(Fl)pS-pl-K(Dab)G-NH_2 (**5**) were synthesized using modified Fmoc solid-phase peptide synthesis and purified by reversed-phase HPLC (RP-HPLC) (see Supporting Information for synthesis details). A C-terminal-truncated Smad2 thioester (MH2 domain, residues 241−462) was prepared as previously reported.[4] An excess of SARA (residues 665−721) was added to the protein, and the Smad2/SARA complex was purified by ion-exchange chromatography. The ligation reactions[7] between the thioester and the different peptides were monitored by a combination of RP-HPLC, SDS-PAGE, and mass spectrometry and were found to be complete in 12−24 h. The proteins (labeled **1**−**5** according to the corresponding peptide number, Table 1) were then purified by gel filtration chromatography.

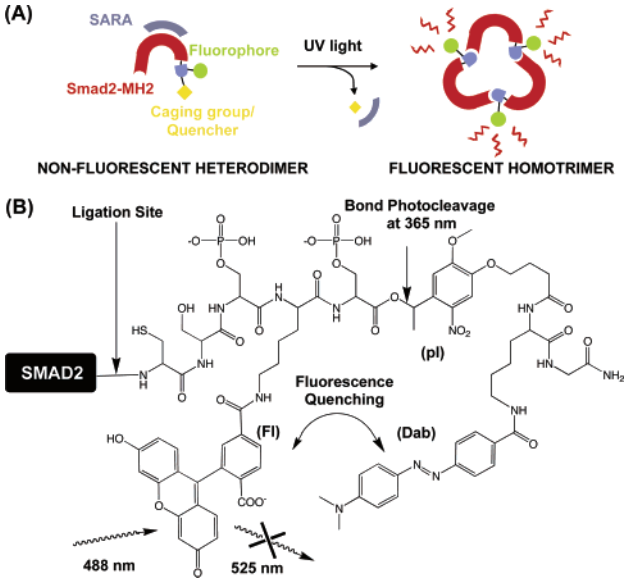

Figure 1. (A) Schematic representation of the simultaneous triggering of Smad2 activity and fluorescence. (B) Design of caged Smad2 (MH2 domain, residues 241−467) based on a modified CSpSMpS peptide.

Preliminary gel filtration and RP-HPLC analyses showed that proteins **3** and **4** were homotrimer and heterodimer,[8] respectively (proteins **1** and **2** were used as controls).[9] Therefore, the fluorescently labeled M466K protein behaves in vitro as the wild-type phosphorylated protein. In contrast, attachment of a bulky caging group at the C-terminus of **4** disrupted its activity (as compared to **2)** even though it is phosphorylated. Importantly, UV irradiation of **4** resulted in the formation of the active homotrimer (protein **6**, Table 1).[10]

After establishing that both protein caging and labeling were independently compatible with our design, we investigated the biophysical properties of the protein containing the caging group, the fluorophore, and the quencher. Before exposure to UV light,

10.1021/ja0499142 CCC: $27.50

Table 1. Semi-Synthetic Proteins and Their Properties

	protein C-terminal sequence	expected/ observed mass (Da)	oligomeric state[a]
1	CSSMS	25358.8 / 25361.7 ± 3.1	heterodimer
2	CSpSMpS	25518.7 / 25526.0 ± 4.3	homotrimer
3	CSpSK(Fl)pS	25874.1 / 25878.0 ± 5.6	homotrimer
4	CSpSMpS-pl-K	25928.2 / 25930.6 ± 4.9	heterodimer
5	CSpSK(Fl)pS-pl-K(Dab)G	26590.8 / 26598.1 ± 4.3	heterodimer
6	CSpSMpS-pl-K + $h\nu$[b]	25518.7 / 25522.3 ± 7.2	homotrimer
7	CSpSK(Fl)pS-pl-K(Dab)G + $h\nu$[b]	25874.1 / 25880.8 ± 2.9	homotrimer

[a] Determined by analytical gel filtration chromatography and RP-HPLC. Homotrimer refers to Smad2 homotrimer, while heterodimer refers to Smad2/SARA heterodimer. [b] Proteins were photolyzed at 325 or 365 nm.

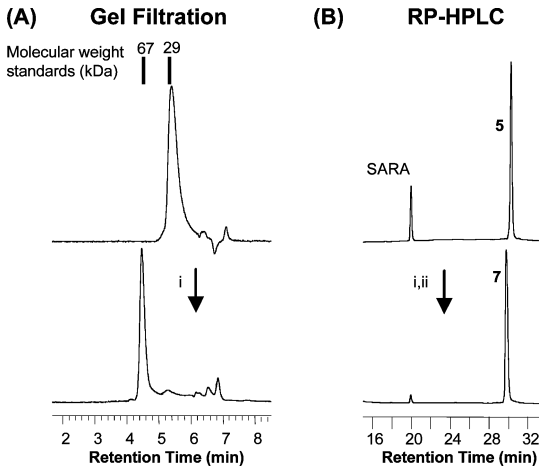

Figure 2. (A) Gel filtration analysis of the oligomeric state of protein **5** before and after irradiation with UV light. (B) RP-HPLC of proteins **5** and **7**: (i) 3 s irradiation at 325 nm with He−Cd laser, (ii) gel filtration chromatography purification.

protein **5** was a heterodimer (Figure 2) and was essentially nonfluorescent (Figure 3), due to efficient intramolecular quenching of fluorescein by dabcyl. Irradiation with UV light resulted in photolinker cleavage and homotrimer formation (Figure 2).[11] Importantly, this was also accompanied by an approximate 26-fold increase in fluorescence (Figure 3).[12] The photolytic conversion was characterized at high and low light intensities[13] by monitoring the fluorescence emission of the product. At low intensity, the photolysis was first order with a rate constant of 4.0×10^{-3} s^{-1} and an observed quantum yield of 0.03. Photolysis was quantitative in less than 3 s when a high-intensity laser was used.[14]

In conclusion, we have shown that protein activity and fluorescence can be triggered simultaneously. By coupling fluorescence to activity, an easily detectable fluorescent signal can be used as a convenient read-out of the phototriggering of protein activity. This general scheme can be used for caging proteins where the C-terminus is vital for biochemical interactions but should also be applicable to the caging of amino acid side chains. We are currently in the process of using the caged protein to study the physical behavior of Smad2 and the kinetics of the TGF-β signaling pathway inside living cells.

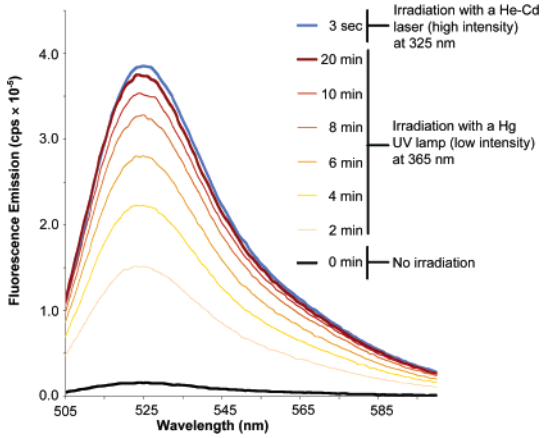

Figure 3. Fluorescence spectra of protein **5** (1 μM) at varying UV irradiation times. Samples were irradiated at either 325 or 365 nm, with irradiances of 4.740 and 0.005 W/cm^2, respectively. Fluorescence excitation was performed at 488 nm.

Acknowledgment. We thank Jennifer Ottesen for helpful suggestions on the preparation of the proteins. This work was supported by the National Health Institute (Grant GM55843-07). M.E.H. was supported by NIH MSTP Grant GM07739.

Supporting Information Available: Full experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Ligation reactions were performed at 4 °C by addition of the chemically synthesized peptides (final concentration of 1 mM) to the protein thioester/SARA complex (300 μM) in a buffer containing 100 mM Hepes (pH 8.0), 200 mM NaCl, and 50 mM 2-mercaptoethanesulfonic acid.

(8) Homotrimer refers to a Smad2 homotrimer (active), while heterodimer refers to a Smad2/SARA heterodimer (inactive).

(9) SARA binding to Smad2 was evaluated by RP-HPLC analysis of the purified ligation products. For example, analysis of **1**/SARA gave a peak area ratio of ~4, indicative of an heterodimer, whereas analysis of **2**/SARA gave a peak area ratio of more than 45 (>92% reduction in SARA signal).

(10) Irradiation of protein **1**, **2**, and **3** at 325 or 365 nm had no effect on their properties.

(11) Experiments were carried out with protein concentrations of 100, 10, and 1 μM. Results obtained were similar over this concentration range.

(12) Irradiation of two model peptides, GEK(Fl)K-pl-K(Dab)G and GEKE-pl-K(Dab)G, demonstrated that apart from fluorescein, no fluorophore or photoproducts contribute to fluorescence emission when the excitation wavelength is 488 nm. UV irradiation of fluorescein under the conditions tested did not result in any noticeable photobleaching of the fluorophore.

(13) Irradiation was performed with either a He−Cd laser (325 nm and 4.74 W/cm^2) or a Hg lamp equipped with a narrow bandwidth filter (365 nm and 5 mW/cm^2).

(14) HPLC analysis of the photoproducts showed 100% conversion. No proteinogenic side products were detected.

JA0499142