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A Light-Activated Probe of Intracellular Protein Kinase Activity

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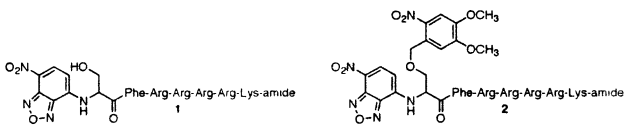
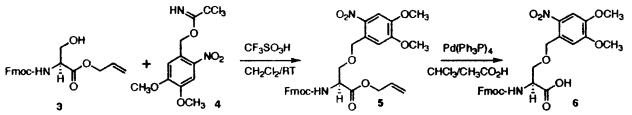
Signal transduction pathways are driven, in large part, by the intracellular action of protein kinases. Members of this large enzyme family (>500) catalyze the transfer of the γ-phosphoryl group of ATP to serine, threonine, and tyrosine residues in a wide variety of protein substrates. These enzymes play an important role in nearly every aspect of normal cellular function. In addition, nearly every human malady has been linked, in some manner, to abnormal protein kinase activity. Not surprisingly, there has been and continues to be intense interest in the development of protein kinase inhibitors as potential therapeutic agents. In addition, several protein kinase sensors have recently been described that furnish a visual readout of protein kinase activity in living cells. These probes not only reveal the activity of protein kinases in response to some environmental stimulus, but they also offer a realistic cell-based assessment of protein kinase inhibitor efficacy. Several investigators have described genetically encoded protein kinase substrates that possess appended green fluorescent protein analogues for visual readout.[1] In addition, we've recently developed several peptide-based species that respond to phosphorylation in a fluorescently sensitive fashion.[2] For example, compound **1**, which contains an environmentally sensitive fluorophore positioned within a few angstroms of the phosphorylatable serine hydroxyl moiety, displays a robust response to protein kinase C (PKC)-catalyzed phosphorylation.[2a] However, none of the fluorescent reporters described to date allow the investigator to control *when* protein kinase activity sampling is performed. The latter property would be extremely valuable in a number of instances. For example, cells harboring constitutively active protein kinases can render the intracellular loading of a kinase sensor and the subsequent observation of activity at a well-defined time point problematic. Furthermore, protein kinases can exhibit intermittent activity as a function of some cellular event, such as with PKC, which appears to be activated at several distinct stages during mitosis.[3] In general, the ability to control when protein kinase activity is measured with respect to multiple cellular signposts provides the opportunity to collect a large series of parallel temporally offset samplings of protein kinase action within the context of a single experiment. We report herein the first example of a light-activated ("caged") probe (**2**) of protein kinase activity.



We envisioned two different strategies for the construction of caged protein kinase sensors. PKC is known to recognize peptides containing appropriately positioned arginine residues.[4] Consequently, a substrate harboring multiply caged arginine moieties[5] should be resistant to PKC-catalyzed phosphorylation until activated by light. Alternatively, the presence of a single photolytically sensitive substituent on the phosphorylatable serine hydroxyl should likewise preclude PKC-catalyzed phosphoryl transfer. The latter offers the advantage, relative to a peptide containing multiply caged residues, that only a single functional group need be photolytically liberated (peptide **2**) to generate the active protein kinase fluorescent reporter. The caged serine was prepared as outlined in Scheme 1.[6] The key step, benzylation of the serine side-chain hydroxyl, was achieved using the trichloroacetimidate **4** in the presence of a catalytic amount of triflic acid.[7] The Fmoc derivative **6** was subsequently employed to create the active site-directed peptide **2** via solid-phase peptide synthesis (see Supporting Information).

Scheme 1



As would be expected for a peptide lacking a free hydroxyl group, compound **2** fails to serve as a substrate for PKC (Figure 1). Two potentially useful attributes of the caged substrate include (1) sampling of protein kinase activity at a time of the investigator's choosing and (2) control over the amount of active substrate available for phosphorylation. The former is illustrated in Figure 1, where the caged substrate **2** is incubated with active PKC for various time intervals (10, 20, and 30 min) and then subsequently photoactivated (Hg arc lamp for 90 s). A broad band-pass filter (300−400 nm with λ_{max} @ 360 nm) was employed to protect the nitrobenzofurazan fluorophore from photobleaching, and an IR filter was used to shield PKC from heat inactivation. Peptide **2** is completely inert as a PKC substrate irrespective of PKC incubation time. Furthermore, identical robust fluorescence responses are immediately observed following photolysis, irrespective of the pre-photolysis PKC incubation time.

HPLC analysis revealed that the maximal conversion of caged to uncaged substrate is approximately 60% (see Supporting Information). The quantum yield for photolytic conversion is 0.06 as determined by actinometry.[8] Although a 90 s irradiation time is required for maximal in vitro formation of the uncaged substrate, intracellular uncaging should proceed more rapidly due to an enhanced photon flux through a comparatively smaller cellular volume. Furthermore, as illustrated in Figure 2, total photon flux can be used to control the amount of free protein kinase probe liberated.

Both the timing and relative amount of sensor release can be controlled in a single experiment (Figure 1, insert). Approximately half of compound **2** was photochemically converted to the active probe **1** in the presence of PKC, as indicated by the observed change in fluorescence. Subsequent illumination of the reaction mixture afforded additional free sensor, which likewise furnished a fluorescent response. These experiments demonstrate that it is not only

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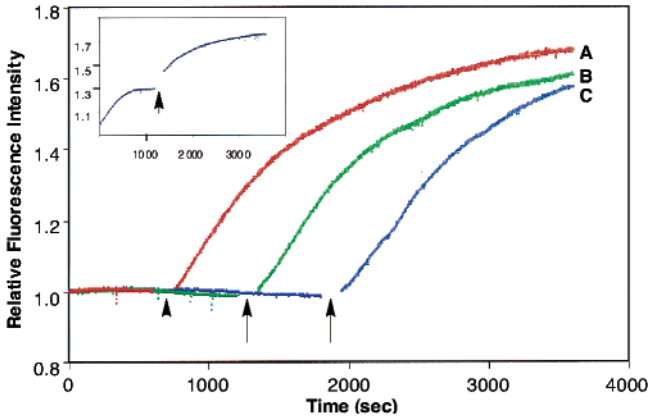

Figure 1. Time-dependent change in fluorescence before and after in situ illumination of caged peptide. The caged peptide **2** was incubated at 30 °C with PKCα and the change in fluorescence measured for 10 (A), 20 (B), or 30 (C) min. Samples were then irradiated at the indicated time points. (Insert) Partial photolysis of **2** followed by a second exposure to brief illumination. See Supporting Information for details.

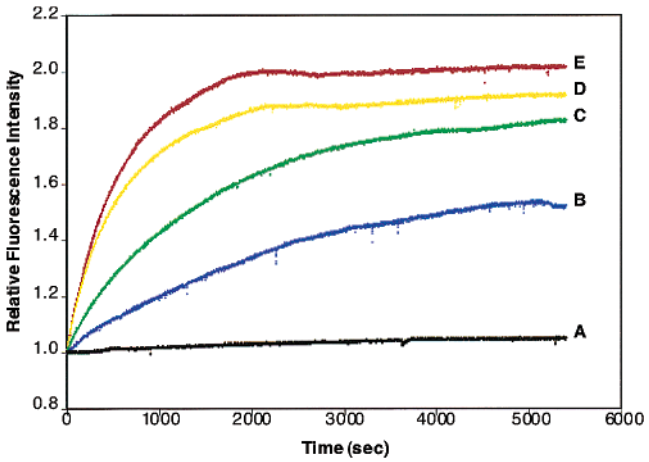

Figure 2. Fluorescence change as a function of irradiation time. Peptide **2** was illuminated for (A) 0, (B) 15, (C) 30, (D) 45, and (E) 90 s and PKCα-catalyzed activity subsequently sampled via fluorescence change.

possible to control the timing of protein kinase activity sampling but also that activity measurements can be performed at multiple stages as a function of cellular events. The latter is noteworthy since it establishes that an inert sensor can be held in reserve so that kinase activity can be assessed at multiple time points.

Finally, we examined the light-induced sampling of protein kinase activity in living cells. The caged protein kinase fluorescent substrate **2** was introduced into HeLa cells via microinjection. Exposure of cells to the phorbol ester TPA activates PKC. We have previously demonstrated that compound **1** serves as a specific sensor for the conventional isoforms of PKC in living cells.[2a] However, HeLa cells containing the caged derivative **2** fail to display a fluorescent response upon exposure to TPA alone or upon exposure to light in the absence of TPA. By contrast, a robust response is observed when compound **2**-containing HeLa cells are both illuminated and treated with TPA (Figure 3).

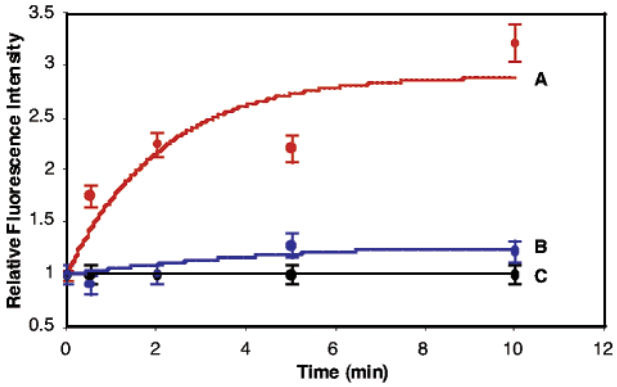

Figure 3. Intracellular fluorescence change as a function of time following irradiation and/or TPA treatment. HeLa cells were microinjected with peptide **2** and subsequently (A) irradiated and treated with TPA, (B) treated with TPA in the absence of light, and (C) irradiated in the absence of TPA

In summary, we have prepared a caged protein kinase sensor via introduction of a serine moiety containing a photolytically labile side-chain appendage. The presence of the latter affords control over both the timing and amount of active sensor release. To the best of our knowledge, compound **2** represents the first example of a caged fluorescent reporter of intracellular enzymatic activity.

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Supporting Information Available: Experimental details of the synthesis, characterization, photo-uncaging, and in vitro and in vivo assays of compound **2** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Nagai, Y.; Miyazaki, M.; Aoki, R.; Zama, R.; Zama, T.; Inouye, S.; Hirose, K.; Lino, M.; Hagiwara, M. *Nat. Biotechnol.* **2000**, *18*, 313−6. (b) Kurokawa, K.; Mochizuki, N.; Ohba, Y.; Mizuno, H.; Miyawaki, A.; Matsuda, M. *J. Biol. Chem.* **2001**, *276*, 31305−10. (c) Zhang, J.; Ma, Y.; Taylor, S. S.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14997−5003. (d) Ting, A. Y.; Kain, K. H.; Klemke, R. L.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 15003−8. (e) Sato, M.; Ozawa, T.; Inukai, K.; Asano, T.; Umezawa, Y. *Nat. Biotechnol.* **2002**, *20*, 287−94.

(2) (a) Yeh, R. H.; Yan, X.; Cammer, M.; Bresnick, A. R.; Lawrence, D. S. *J. Biol. Chem.* **2002**, *277*, 11527−11532. (b) Chen, C.-A.; Yeh, R. H.; Lawrence, D. S. *J. Am. Chem. Soc.* **2002**, *124*, 3840−3841.

(3) (a) Goss, V. L.; Hocevar, B. A.; Thompson, L. J.; Stratton, C. A.; Burns, D. J.; Fields, A. P. *J. Biol. Chem.* **1994**, *269*, 19074−19080. (b) Collas, P. *J. Cell Sci.* **1998**, *111*, 1293−1303. (c) Passalacqua, M.; Patrone, M.; Sparatore, B.; Pedrazzi, M.; Melloni, E.; Pontremoli, S. *FEBS Lett.* **1999**, *453*, 249−253. (d) Takai, Y.; Ogawara, M.; Tomono, Y.; Moritoh, C.; Imajoh-Ohmi, S.; Tsutsumi, O.; Taketani, Y.; Inagaki, M. *J. Cell Biol.* **1996**, *133*, 141−149. (e) Varlamova, O.; Spektor, A.; Bresnick, A. R. *J. Muscle Res. Cell Motil.* **2001**, *22*, 214−250.

(4) Nishikawa, K.; Toker, A.; Johannes, F. J.; Songyang, Z.; Cantley, L. C. *J. Biol. Chem.* **1997**, *272*, 952−960.

(5) Wood, J. S.; Koszelak, M.; Liu, J.; Lawrence, D. S. *J. Am. Chem. Soc.* **1998**, *120*, 7145−7146.

(6) Others have reported the synthesis of side-chain caged serine: (a) Pirrung, M. C.; Nunn, D. S. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1489−92. (b) Cook, S. N.; Jack, W. E.; Xiong, X.; Danley, L. E.; Ellman, J. A.; Schultz, P. G.; Noren, C. J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1629−30.

(7) (a) Iversen, T.; Bundle, D. R. *J. Chem. Soc., Chem. Commun.* **1981**, 1240−1241 (b) Wessel, H.-P.; Iversen, T.; Bundle, D. R. *J. Chem. Soc., Perkin Trans.* **1985**, *1*, 2247−2250.

(8) Allen, J. M.; Allen, S. K.; Baertschi, S. W. *J. Pharm. Biomed. Anal.* **2000**, *24*, 167−178.

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