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## Light-Regulated Sampling of Protein Tyrosine Kinase Activity

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Protein tyrosine kinases (PTKs) are well-established participants in signaling pathways that drive and maintain a host of normal and aberrant cellular phenotypes. The complexities associated with the regulation of PTK activation are most appropriately examined in the intracellular environment, conditions that allow PTK activity to be correlated with cellular behavior. Consequently, there has been intense interest in developing reporters that can fluorescently sense PTK activity in real time and under conditions that are consistent with living cells.<sup>1,2</sup> Recently, we described a strategy for the design of PTK reporters that relies upon the phosphorylation-induced disruption of noncovalent interactions between the kinase-targeted Tyr residue and a proximal pyrene moiety.<sup>3</sup> However, the latter, like all other PTK sensors described to date, is immediately susceptible to phosphorylation. The inability to control sensing activity precludes an assessment of intracellular kinase action as a function of the cell cycle in particular, and environmentally stimulated behaviors in general. We report the first example of a light-regulated protein tyrosine kinase sensor.

Short wavelength fluorophores, such as pyrene, are inappropriate for cell-based studies since cellular autofluorescence results in background interference. With this limitation in mind, we constructed a small peptide library containing several fluorophores positioned on the (L)-2,4-diaminobutanoic acid 1 (Dab) and the (L)-2,3-diaminopropionic acid 2 (Dap) at the Y-2 position and the Y+3 positions, respectively, of a known Src kinase substrate (see Chart 1).<sup>3</sup> Oregon green,<sup>4</sup> cascade blue,<sup>6</sup> and cascade yellow<sup>6</sup> are relatively bright fluorophores with high quantum yields, good resistance to photobleaching, and distinct spectral properties. The cascade yellow Y-2 derivative 3 exhibits a 2.7-fold enhancement in fluorescence intensity upon phosphorylation. By contrast, the corresponding cascade yellow-substituted derivative at Y+3 (i.e., 4) fails to furnish a fluorescence response. In addition, Oregon green (5) and cascade blue (6) display 2-fold enhancements at Y-2 but more modest changes at Y+3.

NOESY experiments on **3** and its phosphorylated counterpart revealed that the Tyr residue in both peptides exhibits through-space interactions with the fluorophore (Supporting Information), with significantly stronger interactions in the nonphosphorylated species. Indeed, chemical shift data demonstrate that the free Tyr phenol in **3** is significantly more shielded relative to Tyr residues in general<sup>7,8</sup> and the phosphoTyr moiety in phospho-**3** in particular. These results suggest that the ring current of the proximal fluorophore electronically shields the Tyr aromatic ring, an interaction disrupted by phosphorylation. Tyr is known to participate in stacking interactions with and quenching by other aromatic moieties.<sup>9,10</sup>

*Chart 1.* General Structures (1, 2) and the Cascade Yellow (3, 4), Oregon Green (5), and Cascade Blue (6) Derivatives of the PTK Reporters



The nature of the fluorophore influences the efficiency of phosphorylation (Supporting Information). The negatively charged cascade blue derivative **6** displays a low  $K_{\rm m}$  value (3.0  $\pm$  0.2  $\mu$ M), which may be a good barometer of enzyme affinity since the Src kinase exhibits a proclivity for negatively charged substrates. By contrast, the partially positively charged cascade yellow derivative 3 exhibits a significantly higher  $K_{\rm m}$  value (300  $\pm$  10  $\mu$ M). The spectral characteristics of these fluorophores should prove useful for sampling the activity of more than one protein kinase or for use in conjunction with other probes. For example, 5 ( $\lambda_{ex} = 495$ nm;  $\lambda_{em} = 520$  nm) and 6 ( $\lambda_{ex} = 400$  nm;  $\lambda_{em} = 422$  nm) are excited by and emit at dramatically different wavelengths. Consequently, two PTKs that phosphorylate different peptide substrates could potentially be simultaneously monitored. By contrast, **3** ( $\lambda_{ex} = 400$ nm;  $\lambda_{em} = 550$  nm) and **6** are excited at a single wavelength, but display different emission properties.

Src kinase activity is regulated via intramolecular interactions between the catalytic core and appended SH2 and SH3 domains.<sup>11</sup> Furthermore, ligands that target SH2 or SH3 domains are known to activate Src and related PTKs.<sup>12</sup> We recently described a series of moderate to high affinity SH3 ligands.<sup>13</sup> In the presence of SH3 ligand **7** (see Chart 2), Src kinase activity is enhanced 2.7-fold in terms of  $V_{\text{max}}$  (4.8 ± 0.6 vs 1.8 ± 0.1  $\mu$ mol/(min•mg)) and nearly 4-fold in terms  $V_{\text{max}}/K_{\text{m}}$ .

We examined the ability of **3** to fluorescently report Src kinase activity in cell lysates. Hek 293 cells were transiently transfected with a plasmid encoding wild type Src kinase. Cells were lysed, the lysate added to a buffer solution of **3**, and ATP subsequently introduced to initiate the reaction. Kinase activity is visualized as a smooth enzyme-catalyzed progress curve [Figure 1A (ii)]. In addition, the SH3 domain ligand **7** accelerates the initial rate by

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Chart 2. The SH3 Domain Ligand 7, Which Activates Src Kinase Activity, and the Caged Tyrosine Kinase Reporter 8



2.4-fold (i), which is consistent with the effect produced by 7 on pure enzyme. By contrast, lysates from nontransfected cells display negligible PTK activity (iv), with only minor activity in the presence of ligand 7 (iii). The latter suggests that endogenous PTK(s) capable of phosphorylating 3 are present, but their intracellular concentration is highly diluted upon addition of lysis buffer.

As noted above, a limitation associated with PTK reporters is their unregulated vulnerability to phosphorylation. PTKs may be constitutively active or may display periods of intermittent activity as a function of some cellular event. The ability to control when the reporter is functional provides a means to define an explicit start point and sample period. With this in mind, we prepared peptide 8, which contains a tyrosine residue that is covalently modified with a photolabile nitrobenzyl substituent. As expected, compound 8 fails to serve as a Src kinase substrate (Figure 1B). Upon photolysis, the nitrobenzyl moiety is lost and the liberated Tyr suffers phosphorylation, which elicits a time-dependent enhancement in fluorescent intensity. In addition to affording exquisite temporal control over sensing activity, limited intermittent photolysis allows the active reporter to be unleashed as a series of discrete packets over time (Figure 1B and Supporting Information). The latter permits multiple sampling experiments in an investigatorcontrolled chronological fashion. Finally, compound 8 was microinjected into A549 cells, a human lung carcinoma cell line expressing high levels of activated Src kinase.<sup>15</sup> Photolysis triggers a time-dependent increase in fluorescent intensity (Figure 2).

In summary, the PTK reporters described in this study contain an array of bright, photochemically stable, fluorophores that display



Figure 1. (A) (i) Reporter 3, SH3 domain ligand 7, and lysate from 293 cells overexpressing Src kinase, (ii) reporter 3 and lysate from 293 cells overexpressing Src kinase, (iii) reporter 3, SH3 domain ligand 7, and lysate from nontransfected 293 cells, (iv) reporter 3 and lysate from nontransfected 293 cells; (B) light-driven conversion of caged reporter 8 to active reporter 3. In the absence of photolysis (t = 0-400 s), no detectable Src kinase activity is observed. Limited photolysis (arrow) at t = 400 s (8 s, Hg arc lamp) generates active sensor which produces a fluorescent response. Subsequent photolysis (arrow) unleashes an additional packet of active sensor.



Figure 2. Light-induced time-dependent change in fluorescence intensity in A549 cells microinjected with the caged tyrosine kinase sensor 8 (estimated final intracellular concentration of 2-20  $\mu$ M). Following microinjection, cells were exposed to UV from a 100 W Hg-Arc lamp through the objective for 8 s to activate the tyrosine kinase sensor. Immediately after UV treatment (A), images were collected at 1 min intervals (Supporting Information for movie). Final image B was obtained at 25 min following activation of the sensor. Fluorescence intensity measurements are relative to control cells containing the caged sensor.

easily differentiated photophysical properties. The change in fluorescence intensity, upon phosphorylation, is consistent with the phosphate-driven disruption of stacking interactions between the fluorophore and the Tyr moiety. Finally, the corresponding caged derivative affords, for the first time, the opportunity to control PTK activity sensing by delivering the active reporter at single or multiple time points over the course of the experiment.

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Supporting Information Available: Experimental details of peptide synthesis and characterization, and enzyme and cell-based assays. Lightinduced time-dependent change in fluorescence intensity in A549 cells microinjected with the caged tyrosine kinase sensor 8 in avi format. This material is available free of charge via the Internet at http:// pubs.acs.org.

## References

- Chen, C. A.; Yeh, R. H.; Yan, X.; Lawrence, D. S. *Biochim. Biophys. Acta* 2004, *1697*, 39–51.
   Rohman, D. M.; Shults, M. D.; Imperiali, B. *Trends Cell Biol.* 2005, *15*,
- 02-510.
- (3) Wang, Q.; Cahill, S. M.; Blumenstein, M.; Lawrence, D. S. J. Am. Chem. Soc. 2006, 128, 1808-1809.
- (4) Sun, W.-C.; Gee, K. R.; Klaubert, D. H.; Haugland, R. P. J. Org. Chem. 1997, 62, 6469-6475.
- Whitaker, J. E.; Haugland, R. P.; Moore, P. L.; Hewitt, P. C.; Reese, M. Anal. Biochem. 1991, 198, 119–130.
- (6) Anderson, M. T.; Baumgarth, N.; Haugland, R. P.; Gerstein, R. M.; Tjioe, T.; Herzenberg, L. A. Cytometry 1998, 33, 435-444.
- (7) Bundi, A.; Wuthrich, K. Biopolymers 1979, 18, 285-298.
- (9) Bundi, M., Walmeli, R. Diobythetis Dry, 16, 205 (2017).
   (8) Bienkiewicz, E. A.; Lumb, K. J. J. Biomol. NMR 1999, 15, 203–206.
   (9) Kraft, B. J.; Masuda, S.; Kikuchi, J.; Dragnea, V.; Tollin, G.; Zaleski, J.
- M.; Bauer, C. E. Biochemistry 2003, 42, 6726-6734.
- (10) Mayer, R.; Toulme, F.; Montenay-Garestier, T.; Helene, C. J. Biol. Chem. **1979**. 254, 75-82
- (11) Boggon, T. J.; Eck, M. J. Oncogene 2004, 23, 7918-7927. (12) Moarefi, I.; LaFevre-Bernt, M.; Sicheri, F.; Huse, M.; Lee, C. H.; Kuriyan, J.; Miller, W. T. *Nature* **1997**, *385*, 650–653.
  (13) Li, H.; Lawrence, D. S. *Chem. Biol.* **2005**, *12*, 905–912.
- (14) Veldhuyzen, W. F.; Nguyen, Q.; McMaster, G.; Lawrence, D. S. J. Am.
- *Chem. Soc.* **2003**, *125*, 13358–13359. Zheng, R.; Yano, S.; Matsumori, Y.; Nakataki, E.; Muguruma, H.; (15)
- Yoshizumi, M.; Sone, S. Clin. Exp. Metastasis 2005, 22, 195-204.

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