DOI: 10.1002/cbic.201000234

Visualizing Tyrosine Kinase Activity with Bipartite Tetracysteine Display

Sarmistha Ray-Saha[b] and Alanna Schepartz*[a]

There is great interest in the development of new tools that can monitor protein kinase activity in living cells, in large part because of the central role of protein phosphorylation in cell signaling. Synthetic peptide-based sensors[1] can be effective in vitro but require special means to gain cell entry.[2] In contrast, fluorescent protein (FP)-based sensors[3] are readily expressed in cells, but their use is complicated by bulk and unpredictability.[4–6] Profluorescent biarsenicals such as FlAsH[7] and ReAsH[8] offer a genetically encoded complement to FPs for detecting and tracking cellular events.[9] These membrane permeable dyes are dark in the ethanedithiol (EDT)-bound form, but become fluorescent upon thiol exchange with proteins that contain a tetracysteine motif. Recently we reported that the linear tetracysteine sequence preferred by FlAsH[7] and ReAsH[8] could be split between two members of a protein partnership or two approximated regions of a folded protein while maintaining high affinity and brightness in vitro and in live cells.[10,11] Here we show that this finding, a tool we refer to as bipartite tetracysteine display, facilitates the design of a first-generation Src family kinase sensor that is both encodable and site-selective. This new sensor design reports on kinase activity as an increase in fluorescence emission in a way that complements existing FP-based biosensors (Figure 1).

Our design began with the X-ray structure of the p130Cas·Lck SH2 complex.[12] We interrogated the interface between the SH2 and phosphotyrosine-containing p130Cas domains (Figure 2 A) to identify adjacent residues that could reproduce a ReAsH binding motif when changed to a bipartite Cys–Cys pair.[13] We focused on substitutions in loops, rather than helices or sheets, as we have shown previously that these locations often yield higher-affinity ReAsH binding sites.[11] After phosphorylation data,[14] mutagenesis studies,[15] and biophysical analyses[12,16] available for the Src SH2 domain and peptide substrate had been considered, 12 potential sensors (Figure S1 in the Supporting Information) were designed. In these designs, one Cys–Cys motif is located between residues 89 to 96 of the homologous Src SH2 domain[17] and the other

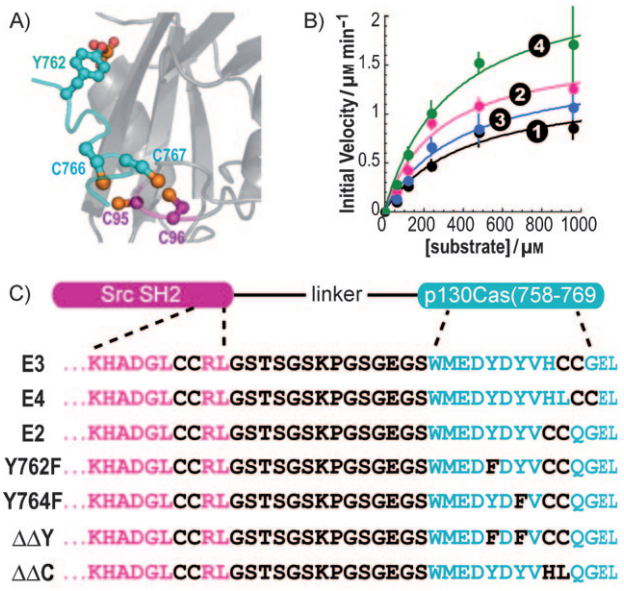


Figure 2. A) Close-up of the interface between Lck SH2 (gray) and p130Cas (cyan) (PDB ID: 1X27); Cys residues were introduced in silico by using PyMOL.[23] B) Initial rates of phosphorylation of **1**–**4** by Src. Values represent the average of three trials ± standard deviation. C) Sequences of E2, E3, E4, and several E2 variants.

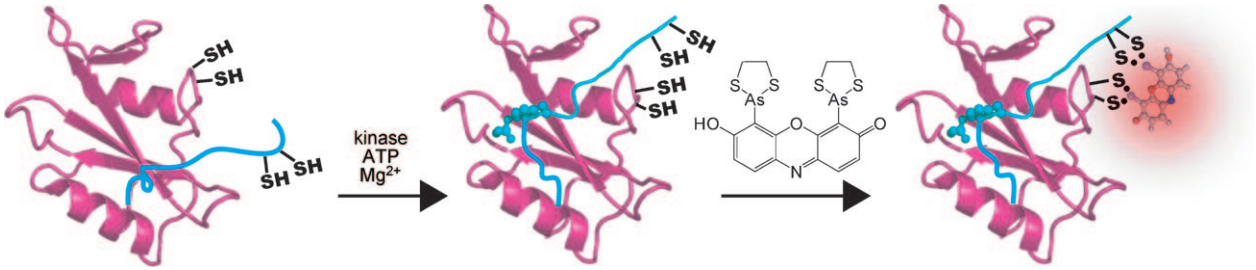


Figure 1. Cartoon illustrating the phosphorylation-dependent change in conformation (step 1) and subsequent ReAsH binding (step 2) by a Src-family-kinase sensor.

[a] *Prof. A. Schepartz*
Departments of Chemistry and Molecular Cellular and Developmental Biology
Yale University
225 Prospect Street, New Haven, CT 06511 (USA)
Fax: (+1) 203-432-3486
E-mail: alanna.schepartz@yale.edu

[b] *S. Ray-Saha*
Department of Molecular Biophysics and Biochemistry, Yale University
260 Whitney Avenue, New Haven, CT 06520 (USA)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201000234.

is located between residues 766 to 769 of p130Cas, immediately beyond the substrate tyrosine. We hypothesized that phosphorylation at Y762 (or Y764, vide infra) would facilitate intramolecular association of the phosphotyrosine with the adjacent SH2 domain; the resulting conformational change would convert an unfolded sensor with low ReAsH affinity into a folded sensor that would bind ReAsH and light up (Figure 1).[18]

To begin, we sought to confirm that the peptide portions of the designed sensors would function as c-$Src_{251-533}$ (Src) substrates. Src activity was evaluated by using an assay in which ATP hydrolysis is linked to NADH oxidation.[19] K_m and k_{cat} values were calculated from plots of initial rates as a function of peptide concentration (Figure 2B). The assay was validated by using a peptide containing the optimized Src substrate (AEEEIYGEFEAKKKKG);[20] the measured K_m (105 ± 26 μM) and k_{cat} (38 ± 4.8 s^{-1}) compare well with values reported for the same substrate.[20] The K_m values of peptides **1** (WMEDYDYVHLQGEL), **2** (WMEDYDYVCCQGEL), **3** (WMEDYDYVHCCGEL), and **4** (WMEDYDYVHLCCEL) range from 285 to 446 μM, and the k_{cat} values range from 2.2 to 4.3 s^{-1} (Table S2). These values suggested that all of our potential sensors should function as Src kinase substrates.

Three of the 12 potential sensors (E2, E3, and E4; Figure 2C) were purified readily from *E. coli* lysates and evaluated to determine if they were Src substrates. Each potential sensor (50–70 μM) was incubated with 0.2 μM Src for 1 h in the presence and absence of ATP. After removal of Mg^{2+} and ATP, the extent of phosphorylation was analyzed by using Western blots and MALDI-MS following digestion with trypsin. Western blots probed with an anti-phosphotyrosine antibody confirmed that E2, E3, and E4 were phosphorylated under these conditions, and MALDI-MS verified an 80 Da increase in mass of the expected tryptic fragment only in the presence of ATP (Figures S3 and S4). Together these results indicate that E2, E3, and E4 are acceptable Src substrates.[21]

We next turned to fluorescence spectroscopy to evaluate if the potential sensors would bind to ReAsH with high affinity and in a phosphorylation-dependent fashion. The phosphorylated and non-phosphorylated forms of E2, E3, and E4 were incubated with ReAsH (25 nM) and EDT (1 mM), and the fluorescence emission at 630 nm was monitored as a function of sensor concentration (Figure 3A) and time (Figure S5). Only phosphorylated E2 displayed a measurable affinity for ReAsH ($K_{app} \sim 8.4 \pm 1.3$ μM); the fluorescence emission observed after treatment of E2 with Src, ATP, and Mg^{2+} was 77% higher than when E2 was treated with Src and Mg^{2+}. This fluorescence-intensity increase is comparable to that observed for simpler bipartite tetracysteine motifs.[11] Neither E3 nor E4 displayed a detectable affinity for ReAsH whether phosphorylated or not (Figure 3A). The increase in the fluorescence emission of E2 upon phosphorylation is higher than that of all but one of the FRET-based Src kinase sensors reported in the literature.[6,18,22] Even more importantly, the binding of ReAsH by the phosphorylated form of E2 results in a straightforward readout of kinase activity as an increase in fluorescence emission, without the need for multiple measurements to determine FRET ratios.[4,18]

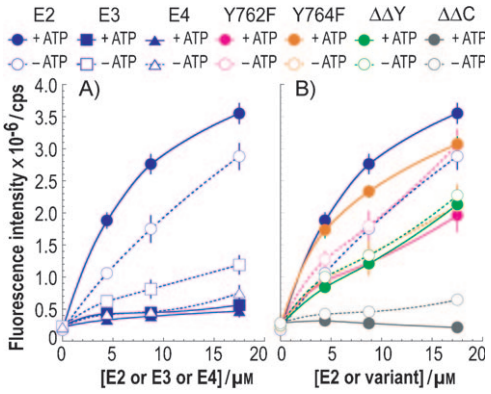


Figure 3. Fluorescence emission at 630 nm of 25 nM ReAsH after a 60-min incubation with phosphorylated (+ATP) or non-phosphorylated (-ATP) A) E2, E3 and E4 or B) E2 and variants thereof. Values represent the average of three trials ± standard deviation.

A series of experiments were performed to better understand the origins of the phosphorylation-dependent change in ReAsH affinity for E2. The substrate region of E2, which is derived from p130Cas, contains two tyrosines, at positions 762 and 764, and questions remain regarding the role of each in mediating downstream, phosphorylation-dependent events. Previously reported FRET-based sensors respond to phosphorylation at both positions; this makes it difficult to monitor phosphorylation in a site-selective manner.[18] To determine whether the design of E2 would allow it to discriminate between phosphorylation at positions 762 or 764, we expressed three E2 variants in which one or both tyrosines were substituted by phenylalanine (Y762F, Y764F and ΔΔY; Figure 2C). Each E2 variant was treated with Src and Mg^{2+} in the presence and absence of ATP as described; MALDI-MS and Western-blot analysis confirmed that Y762F and Y764F were phosphorylated, whereas ΔΔY was not (Figures S3, S4). With no substrate tyrosines, ΔΔY also showed poor affinity for ReAsH irrespective of treatment with ATP (Figure 3B). The phosphorylated form of Y764F bound ReAsH with an affinity that rivaled that of phosphorylated E2 (Figure 3B) and underwent a comparably large increase in fluorescence relative to the non-phosphorylated form (78%). In contrast, Y762F bound ReAsH poorly whether phosphorylated or not. These results suggest that the increase in ReAsH affinity and brightness observed with E2 results from phosphorylation at position 762, as predicted by our design. Thus, unlike FRET-based sensors, E2 is sensitive only to phosphorylation at Y762. As expected, an E2 variant containing only one Cys–Cys pair (ΔΔC, Figure 2C) showed minimal affinity for ReAsH and was not bright even upon phosphorylation (Figure 3B).

Finally, we set out to evaluate the extent to which E2 would function as a substrate for kinases within and outside the Src family. We incubated E2 with ATP and Mg^{2+} along with 0.025 units of Src family kinases Src, Yes, Lck, and Fyn as well as the non-Src family kinases JAK2 and PKA. Western-blot analysis of E2 after incubation with these kinases indicated that it is an effective substrate for phosphorylation by Src and Yes but not Lck, Fyn, JAK2, and PKA (Figure S6).

In summary, we have reported that bipartite tetracysteine display[10,11] can facilitate the design of a first-generation Src-family-kinase sensor that is both encodable and site-selective. Binding of ReAsH by phosphorylated E2 leads to an increase in fluorescence in vitro that is at least as high as that observed with simpler bipartite tetracysteine motifs, and higher than all but one of the reported FRET-based Src kinase sensors. As an additional advantage, the binding of ReAsH by the phosphorylated form of E2 results in a straightforward readout of kinase activity as an increase in fluorescence emission. Finally, unlike FRET-based Src kinase sensors, which respond to phosphorylation at both Y762 and Y764, E2 responds only to phosphorylation at Y762. This observation suggests that more fully optimized versions of E2—optimized with respect to ReAsH affinity, brightness, and kinase selectivity—could prove useful in visualizing position-dependent phosphorylation events in living cells.

Experimental Section

Please see the Supporting Information for experimental details.

Acknowledgements

We are grateful to the NIGMS for support (GM 83257), and to Jennifer S. Griffiths, Laura Strittmatter, and Harvey Chin for aid with experimental design and/or cloning. c-Src$_{251-533}$ and YopH phosphatase were gifts from John Kuriyan.

Keywords: fluorescence · phosphorylation · ReAsH · sensors · tyrosine kinases

[1] a) E. Luković, J. A. González-Vera, B. Imperiali, *J. Am. Chem. Soc.* **2008**, *130*, 12821 – 12827; b) E. Luković, E. V. Taylor, B. Imperiali, *Angew. Chem.* **2009**, *121*, 6960 – 6963; *Angew. Chem. Int. Ed.* **2009**, *48*, 6828 – 6831; c) V. Sharma, Q. Wang, D. S. Lawrence, *Biochim. Biophys. Acta Proteins Proteomics* **2008**, *1784*, 94 – 99.

[2] D. S. Lawrence, Q. Z. Wang, *ChemBioChem* **2007**, *8*, 373 – 378.

[3] a) N. N. Aye-Han, Q. Ni, J. Zhang, *Curr. Opin. Chem. Biol.* **2009**, *13*, 392 – 397; b) W. B. Frommer, M. W. Davidson, R. E. Campbell, *Chem. Soc. Rev.* **2009**, *38*, 2833 – 2841; c) K. J. Herbst, Q. Ni, J. Zhang, *IUBMB Life* **2009**, *61*, 902 – 908.

[4] R. Y. Tsien, *Annu. Rev. Biochem.* **1998**, *67*, 509 – 544.

[5] a) D. M. Chudakov, S. Lukyanov, K. A. Lukyanov, *Trends Biotechnol.* **2005**, *23*, 605 – 613; b) M. T. Kunkel, Q. Ni, R. Y. Tsien, J. Zhang, A. C. Newton, *J. Biol. Chem.* **2005**, *280*, 5581 – 5587.

[6] A. Y. Ting, K. H. Kain, R. L. Klemke, R. Y. Tsien, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 15003 – 15008.

[7] B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **1998**, *281*, 269 – 272.

[8] S. R. Adams, R. E. Campbell, L. A. Gross, B. R. Martin, G. K. Walkup, Y. Yao, J. Llopis, R. Y. Tsien, *J. Am. Chem. Soc.* **2002**, *124*, 6063 – 6076.

[9] a) G. M. Gaietta, B. N. G. Giepmans, T. J. Deerinck, W. B. Smith, L. Ngan, J. Llopis, S. R. Adams, R. Y. Tsien, M. H. Ellisman, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17777 – 17782; b) G. Gaietta, T. J. Deerinck, S. R. Adams, J. Bouwer, O. Tour, D. W. Laird, G. E. Sosinsky, R. Y. Tsien, M. H. Ellisman, *Science* **2002**, *296*, 503 – 507.

[10] N. W. Luedtke, R. J. Dexter, D. B. Fried, A. Schepartz, *Nat. Chem. Biol.* **2007**, *3*, 779 – 784.

[11] J. L. Goodman, D. B. Fried, A. Schepartz, *ChemBioChem* **2009**, *10*, 1644 – 1647.

[12] F. Nasertorabi, K. Tars, K. Becherer, R. Kodandapani, L. Liljas, K. Vuori, K. R. Ely, *J. Mol. Recognit.* **2006**, *19*, 30 – 38.

[13] F. Madani, J. Lind, P. Damberg, S. R. Adams, R. Y. Tsien, A. O. Graslund, *J. Am. Chem. Soc.* **2009**, *131*, 4613 – 4615.

[14] T. Nakamoto, R. Sakai, K. Ozawa, Y. Yazaki, H. Hirai, *J. Biol. Chem.* **1996**, *271*, 8959 – 8965.

[15] J. M. Bradshaw, V. Mitaxov, G. Waksman, *J. Mol. Biol.* **2000**, *299*, 523 – 537.

[16] Z. Songyang, S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, *Cell* **1993**, *72*, 767 – 778.

[17] The SH2 domains of Lck (PDB ID: 1X27) and Src (PDB ID: 1SHA) are 52.9% identical; the Cα atoms align with an RMSD of 1.00711 Å (calculated by using WinCoot v0.6).

[18] Y. Wang, E. L. Botvinick, Y. Zhao, M. W. Berns, S. Usami, R. Y. Tsien, S. Chien, *Nature* **2005**, *434*, 1040 – 1045.

[19] a) S. C. Barker, D. B. Kassel, D. Weigl, X. Huang, M. A. Luther, W. B. Knight, *Biochemistry* **1995**, *34*, 14843 – 14851; b) M. A. Seeliger, M. Young, M. N. Henderson, P. Pellicena, D. S. King, A. M. Falick, J. Kuriyan, *Protein Sci.* **2005**, *14*, 3135 – 3139; c) P. Pellicena, W. T. Miller, *J. Biol. Chem.* **2001**, *276*, 28190 – 28196.

[20] Z. Songyang, K. L. Carraway, M. J. Eck, S. C. Harrison, R. A. Feldman, M. Mohammadi, J. Schlessinger, S. R. Hubbard, D. P. Smith, C. Eng, M. J. Lorenzo, B. A. J. Ponder, B. J. Mayer, L. C. Cantley, *Nature* **1995**, *373*, 536 – 539.

[21] MALDI-MS also detected an 80 Da mass increase upon phosphorylation of E2.

[22] M. X. Ouyang, J. Sun, S. Chien, Y. X. Wang, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 14353 – 14358.

[23] W. L. DeLano, DeLano Scientific, **2002**, San Carlos, CA, USA.

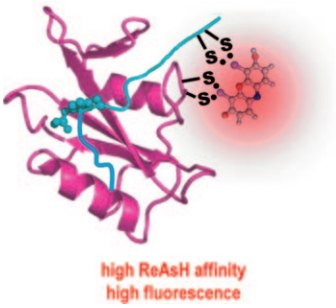
Received: April 14, 2010
Published online on ■■ ■■, 2010

COMMUNICATIONS

*S. Ray-Saha, A. Schepartz**

■■ – ■■

Visualizing Tyrosine Kinase Activity with Bipartite Tetracysteine Display



BiAs binding: Recently we reported that the linear tetracysteine sequence preferred by FlAsH and ReAsH could be split between two members of a protein partnership or regions of a folded protein, while maintaining high affinity and brightness. Here we show that this tool—bipartite tetracysteine display—facilitates the design of E2, an encodable, site-selective, Src-family kinase sensor.

