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Visualizing tyrosine kinase activity with bipartite tetracysteine display

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Abstract

Recently we reported that the linear tetracysteine sequence preferred by FIAsh and ReAsH could be split between two members of a protein partnership or two approximated 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.

Keywords

fluorescence; phosphorylation; ReAsH; sensors; tyrosine kinases

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] Pro-fluorescent biarsenicals such as FIAsh[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 FIAsh[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 tool–bipartite tetracysteine display–facilitates the design of a first-generation Src family kinase sensor that is both encodable and site-selective. This new scaffold has the potential to visualize 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 2A) to identify adjacent residues that could recapitulate a ReAsH binding motif when changed to a bipartite Cys-Cys pair.[13] We focused on substitutions in loops,

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Experimental Section

Please refer to the supporting information for experimental details.

rather than helices or sheets, as we have shown previously that these locations often yield higher affinity ReAsH binding sites.[11] Twelve potential sensors (Figure S1) were designed after considering phosphorylation data,[14] mutagenesis studies,[15] and biophysical analyses[12,16] available for the Src SH2 domain and peptide substrate. In these designs, one Cys-Cys motif is located between residues 89 to 96 of the homologous Src SH2 domain[17] and the other 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-Src₂₅₁₋₅₃₃ (Src) substrates. Src activity was evaluated 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 using a peptide containing the optimized Src substrate (AEEIYGEFEAKKKKG); [20] the measured K_m ($105 \pm 26 \mu\text{M}$) and k_{cat} ($38 \pm 4.8 \text{ s}^{-1}$) compare well with values reported for the same substrate.[20] The K_m of peptides 1 (WMEDYDYVHLQGEL), 2 (WMEDYDYVCCQEL), 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 indicate that all potential sensors should function as Src kinase substrates.

Three of the twelve potential sensors (E2, E3 and E4; Figure 2C) purified readily from *E. coli* lysates and were 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 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 (Figure S3, 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 ($K_{app} \sim 8.4 \pm 1.3 \mu\text{M}$); 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 or 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 all but one FRET-based Src kinase sensor 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]

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, which makes them incapable of monitoring 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 $\Delta\Delta Y$; 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 $\Delta\Delta Y$ was not (Figure S3, S4). With no substrate tyrosines, $\Delta\Delta Y$ also showed poor affinity for ReAsH irrespective of treatment with ATP (Figure 3B). The phosphorylated form of Y764F bound ReAsH with an affinity that rivalled 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 ($\Delta\Delta C$, 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 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, here we report 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 reported FRET-based Src kinase sensor. 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.

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References

1. a Lukovic E, Gonzalez-Vera JA, Imperiali B. J. Am. Chem. Soc. 2008; 130:12821–12827. [PubMed: 18759402] b Lukovic E, Taylor EV, Imperiali B. Angew. Chem.-Int. Edit. 2009; 48:6828–6831. c Sharma V, Wang Q, Lawrence DS. BBA-Proteins Proteomics. 2008; 1784:94–99.
2. Lawrence DS, Wang QZ. ChemBioChem. 2007; 8:373–378. [PubMed: 17243187]
3. a Aye-Han NN, Ni Q, Zhang J. Curr. Opin. Chem. Biol. 2009; 13:392–397. [PubMed: 19682946] b Frommer WB, Davidson MW, Campbell RE. Chem. Soc. Rev. 2009; 38:2833–2841. [PubMed: 19771330] c Herbst KJ, Ni Q, Zhang J. Iubmb Life. 2009; 61:902–908. [PubMed: 19603514]
4. Tsien RY. Annu. Rev. Biochem. 1998; 67:509–544. [PubMed: 9759496]

5. a Chudakov DM, Lukyanov S, Lukyanov KA. Trends Biotechnol. 2005; 23:605–613. [PubMed: 16269193] b Kunkel MT, Ni Q, Tsien RY, Zhang J, Newton AC. J. Biol. Chem. 2005; 280:5581–5587. [PubMed: 15583002]
6. Ting AY, Kain KH, Klemke RL, Tsien RY. Proc. Natl. Acad. Sci. U. S. A. 2001; 98:15003–15008. [PubMed: 11752449]
7. Griffin BA, Adams SR, Tsien RY. Science. 1998; 281:269–272. [PubMed: 9657724]
8. Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, Yao Y, Llopis J, Tsien RY. J. Am. Chem. Soc. 2002; 124:6063–6076. [PubMed: 12022841]
9. a Gaietta GM, Giepmans BNG, Deerinck TJ, Smith WB, Ngan L, Llopis J, Adams SR, Tsien RY, Ellisman MH. Proc. Natl. Acad. Sci. U. S. A. 2006; 103:17777–17782. [PubMed: 17101980] b Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH. Science. 2002; 296:503–507. [PubMed: 11964472]
10. Luedtke NW, Dexter RJ, Fried DB, Schepartz A. Nat. Chem. Biol. 2007; 3:779–784. [PubMed: 17982447]
11. Goodman JL, Fried DB, Schepartz A. ChemBioChem. 2009; 10:1644–1647. [PubMed: 19533719]
12. Nasertorabi F, Tars K, Becherer K, Kodandapani R, Liljas L, Vuori K, Ely KR. J. Mol. Recognit. 2006; 19:30–38. [PubMed: 16245368]
13. Madani F, Lind J, Damberg P, Adams SR, Tsien RY, Graslund AO. J. Am. Chem. Soc. 2009; 131:4613–4615. [PubMed: 19281235]
14. Nakamoto T, Sakai R, Ozawa K, Yazaki Y, Hirai H. J. Biol. Chem. 1996; 271:8959–8965. [PubMed: 8621540]
15. Bradshaw JM, Mitaxov V, Waksman G. J. Mol. Biol. 2000; 299:521–535. [PubMed: 10860756]
16. Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, et al. Cell. 1993; 72:767–778. [PubMed: 7680959]
17. The SH2 domains of Lck (PDB 1X27) and Src (PDB 1SHA) are 52.9% identical; the Ca atoms align with an RMSD of 1.00711 Å (calculated using WinCoot v0.6).
18. Wang Y, Botvinick EL, Zhao Y, Berns MW, Usami S, Tsien RY, Chien S. Nature. 2005; 434:1040–1045. [PubMed: 15846350]
19. a Barker SC, Kassel DB, Weigl D, Huang X, Luther MA, Knight WB. Biochemistry. 1995; 34:14843–14851. [PubMed: 7578094] b Seeliger MA, Young M, Henderson MN, Pellicena P, King DS, Falick AM, Kuriyan J. Protein Sci. 2005; 14:3135–3139. [PubMed: 16260764] c Pellicena P, Miller WT. J. Biol. Chem. 2001; 276:28190–28196. [PubMed: 11389136]
20. Songyang Z, Carraway KL, Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Hubbard SR, Smith DP, Eng C, Lorenzo MJ, Ponder BAJ, Mayer BJ, Cantley LC. Nature. 1995; 373:536–539. [PubMed: 7845468]
21. MALDI-MS also detected an 80 Da mass increase upon phosphorylation of E2
22. Ouyang MX, Sun J, Chien S, Wang YX. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:14353–14358. [PubMed: 18799748]
23. DeLano, WL. DeLano Scientific. San Carlos, CA, USA: 2002.

Figure 2.

(A) Close-up of the interface between Lck SH2 (gray) and p130Cas (cyan) (PDB 1x27); Cys residues were introduced in silico using PyMOL.[23] (B) Initial rates of phosphorylation of 1-4 by Src. Values represent the average of three trials $\pm$ standard deviation. (C) Sequences of E2, E3, E4, and several E2 variants.

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 $\pm$ standard deviation