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Fluorescent Monitoring of Kinase Activity in Real Time: Development of a Robust Fluorescence-Based Assay for Abl Tyrosine Kinase Activity

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Abstract—Fluorescent biosensors hold great promise for drug discovery. Using a solid-phase version of protein semi-synthesis, we incorporated two fluorophores at specific sites within a truncated version of the c-Crk-II protein. The resulting fluorescent protein biosensor permits the real-time monitoring of Abl kinase activity and provides a robust and rapid method for assaying Abl kinase inhibitors.

Deregulation of protein kinases has been implicated in the development of many disease states, making many of these proteins important targets in the field of drug discovery.[1] Indeed, the importance of this area of pharmaceutical research is underscored by the recent approval by the FDA of the Abl tyrosine kinase inhibitor, STI-571, for the treatment of chronic myeloid leukemia (CML).[2–4] Current methods for screening small molecule inhibitors of protein kinases, such as STI-571, typically use ^{32}P phosphotransfer assays,[5] which are expensive, often time consuming and create obvious safety issues. Consequently, nonradioactive kinase assays that allow potential inhibitors to be rapidly screened are likely to be of significant value in this area of drug discovery. Recently, we introduced a fluorescence-resonance energy transfer (FRET) based assay that allowed protein phosphorylation to be directly coupled to a change in fluorescence.[6] Specifically, a dual-labeled version of the adaptor protein, c-Crk-II, was shown to biosense, through a fluorescence change, its own phosphorylation by the Abl tyrosine kinase. Although this work validated the general assay design, the actual change in FRET upon Crk phosphorylation was modest (~3%), thus limiting the utility of this initial biosensor as a screening tool. In the present work, we have generated a second generation Crk biosensor with an improved dynamic range over the original. This reagent permits the real-time monitoring of Abl kinase activity and will provide a robust and rapid method for assaying Abl kinase inhibitors.

c-Crk-II is an intracellular adaptor protein which is composed of an N-terminal Src homology 2 (SH2) domain followed by two SH3 domains through which it mediates a variety of protein–protein interactions in cellular signaling pathways.[7–9] Two protein tyrosine kinases, c-Abl and the epidermal growth factor receptor (EGFR), are known to phosphorylate c-Crk-II on a unique Tyr residue (Tyr221) located between the SH3 domains.[7,10] This post-translational modification is thought to regulate Crk function by inducing an intramolecular association with the SH2 domain.[11,12] Previously, we showed this phosphorylation-triggered conformational change in c-Crk-II could be monitored by FRET when tetramethylrhodamine (Rh) and fluorescein (Fl) probes were incorporated at the N- and C-termini, respectively, of the protein.[6] These studies revealed that phosphorylation of Tyr221 led to a rather small increase in the net distance between the termini (i.e., the fluorophores). (Note, a similar observation has been made very recently using a GFP-based Crk biosensor[13]). This finding was rather unexpected since it implies that unphosphorylated c-Crk-II has a more

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compact domain architecture than previously thought. This idea is further supported by recent mutagenesis studies that indicate the C-terminal SH3 domain acts as a negative regulator of c-Crk-II function, most likely by engaging another part of the protein.[14] These observations provide the framework for the design of a second generation c-Crk-II biosensor which lacks the C-terminal SH3 domain, and thus might be expected to have a less constrained conformation in the unphosphorylated state.

Our design called for the preparation of a dual-labeled c-Crk-II analogue in which the Rh and Fl fluorophores were incorporated at the N- and C-termini of a truncated version of the protein corresponding to residues 2–228. This construct lacks the C-terminal SH3 domain and much of the adjacent linker region. As shown in Figure 1A, the target molecule was prepared using the previously described solid-phase version of Expressed Protein Ligation (EPL).[6] A recombinant c-Crk-II fragment was expressed in *Escherichia coli* as a fusion protein in which the c-Crk sequence was nested between the N-terminal motif MASDLIEGRC- (underlined is a factor Xa recognition site) and a C-terminal yeast VMA intein.[15] Thiolysis of the intein allowed introduction of an α-thioester group into the c-Crk-II fragment which was chemically ligated in situ to synthetic peptide **1** containing both a Fl and a biotin moiety. The latter allowed the ligation product to be immobilized on monomeric-avidin beads. Treatment of the loaded beads with factor Xa unveiled the cryptic N-terminal Cys residue in the protein, thereby facilitating the second ligation reaction with Rh-containing peptide **2**. Following elution from the monomeric avidin beads and further purification, the desired dual-labeled product (hereafter referred to as Rh-Crk△-Fl) was characterized by electrospray mass spectrometry (Fig. 1B), and fluorescence spectroscopy (Fig. 1C). The isolated semi-synthetic material was >95% homogeneous as indicated by SDS-PAGE analysis.

Purified Rh-CrkΔ-Fl was assayed for its ability to biosense its own phosphorylation on Tyr221. Rh-CrkΔ-Fl was treated with full-length recombinant c-Abl kinase[16] and the change in the emission ratio of fluorescein to rhodamine (580:520 nm) was monitored over time. In the presence of ATP, but not in its absence, the emission ratio decreased (indicating a decrease in FRET) over time up to a maximum of around 20% (Fig. 2A). As expected, this change in FRET correlates with phosphorylation of CrkII on Y221 as indicated by Western-blot analysis of time-points of the reaction with anti-phosphotyrosine antibodies (Fig. 2B). Importantly, the observed change in the emission ratio for Rh-CrkΔ-Fl is substantially larger than for the previous construct (20% vs 3%). This result provides further indirect evidence that the C-terminal region of c-Crk-II plays a role in constraining the inter-domain orientations of the protein.

As noted earlier, Kurokawa and co-workers recently described a series of c-Crk-II biosensors related to our own design but containing analogues of the green fluorescent protein (CFP and YFP) appended to either side of core Crk sequence.[13] Consistent with our own

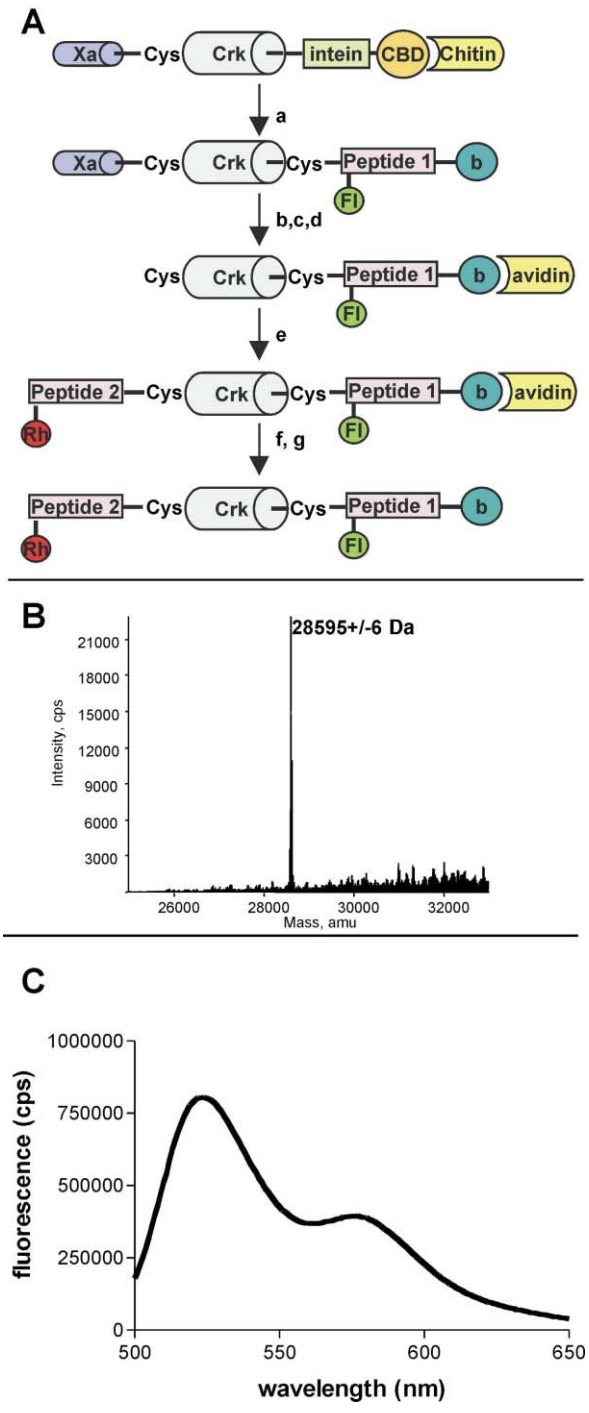

Figure 1. Synthesis and characterization of the Rh-CrkΔ-Fl biosensor. (A) Synthesis of Rh-CrkΔ-Fl by solid-phase EPL. (a) 200 mM NaCl, 60 mM potassium phosphate pH 7.3, 0.1% Triton X-100, 2% (wt/v) 2-mercaptoethane sulfonic acid (MESNA), 5 mg peptide **1** (0.5 mM), 3% (v/v) ethanethiol, 48 h, room temperature (yield 90%); (b) HR Superdex 75 gel filtration chromatography; (c) Monomeric avidin affinity chromatography; (d) PBS (200 mM NaCl, 200 mM potassium phosphate, pH 7.3), 100 units Factor Xa, 2 h, room temperature; (e) PBS, 0.1% Triton X-100, 2% (wt/v) MESNA, 6.8 mg peptide **2** (1 mM); (f) 2 mM biotin, 1 mM dithiothreitol, 0.1% Triton X-100, 1 mM EDTA, 140 mM NaCl, 50 mM Tris, pH 7 (overall yield steps b–f = 18%); (g) HR Superdex 75 gel filtration chromatography. Peptide **1** = H-CGK [Dapa(Fl)]-GLEVLFQGPVRKG-[K$^{\varepsilon}$-biotin)]-G-NH_2. Peptide **2** = tetramethylrhodamine-KRG-propionamide $^{\alpha}$thioester; (B) Electrospray MS of Rh-CrkΔ-Fl. Calculated mass: 28601.0 Da (average isotope comp). (C) Fluorescence emission scan of Rh-CrkΔ-Fl upon excitation at 490 nm.

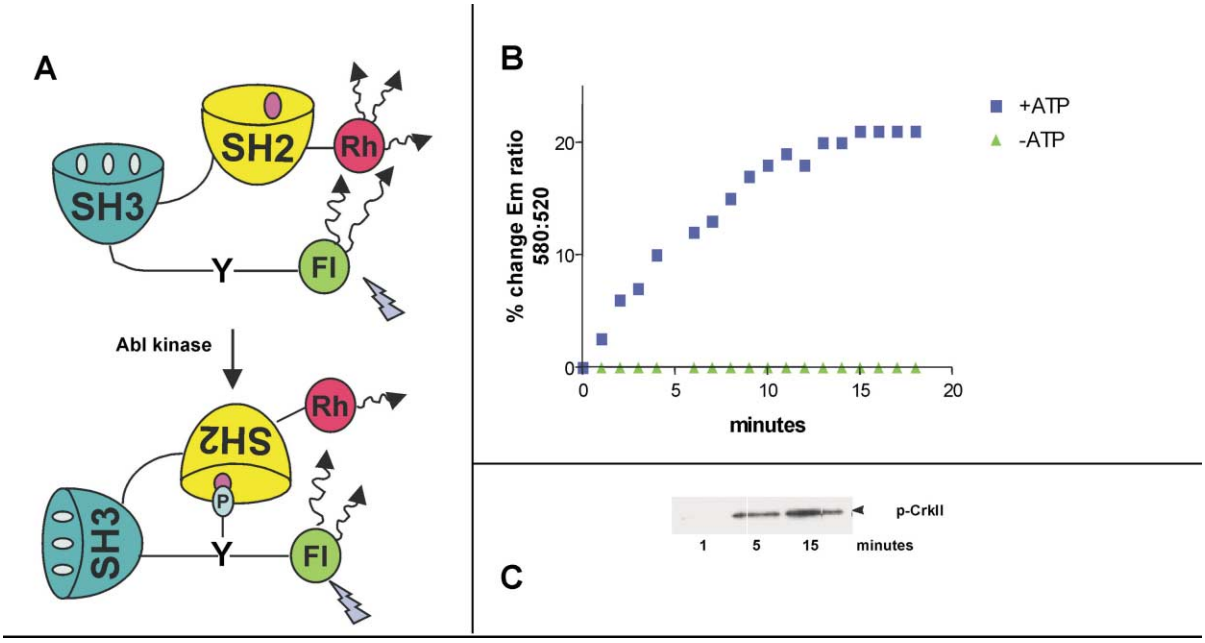

Figure 2. Real-time fluorescence-based Abl-kinase assay. (A) Schematic of assay design; (B) Fluorescence change of Rh-CrkΔ-Fl upon treatment with c-Abl and in the presence (squares) or absence (triangles) of ATP; (C) Anti-phosphotyrosine Western blot of the reaction mixture at various time-points.

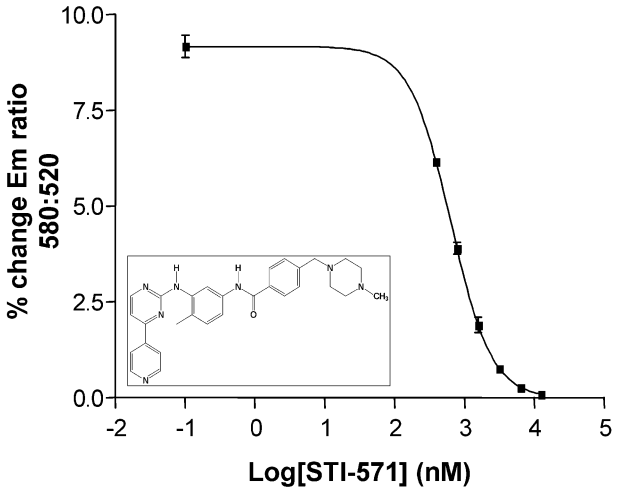

Figure 3. Concentration–response curve for inhibition of Abl kinase activity by STI-571. The structure of the inhibitor is shown in the inset.

results, this group also found that truncated versions of the protein lacking the C-terminal SH3 domain gave larger changes in FRET upon phosphorylation by Abl. Interestingly, phosphorylation of the CFP/YFP biosensor led to an increase in FRET rather than a decrease as observed using Rh-Crk△-Fl. We suspect that this difference may be caused by the use of large protein-based fluorophores in the former versus small molecule fluorophores in the latter biosensor. In the case of the CPP/YFP system, the large size of the fluorophores[17] may result in a more open Crk conformation. This finding perhaps underscores the advantage of using small molecule fluorophores versus GFP analogues when attempting to extract structural information from FRET measurements.

Since Rh-CrkΔ-Fl exhibits a large change in FRET upon phosphorylation, we reasoned that the biosensor should be sensitive to inhibition of Abl kinase activity. To test this idea, we used STI-571, the 2-phenylaminopyrimidine compound developed by Novartis that competitively inhibits the binding of ATP to the Abl kinase in vitro and in vivo.[2,18] Abl kinase reactions were performed in the presence of varying concentrations of STI-571.[19] Use of a fluorescence microtiter plate reader enabled us to compare Abl kinase activity at each concentration of STI-571. The percent change in the emission ratio of each reaction was compared at the same time-point (corresponding to when the control reaction lacking the inhibitor had reached a ~9% change). Figure 3 shows the change in the emission ratio at each concentration of STI-571 and is an average of three independent experiments with each concentration tested in triplicate in each experiment. We found that indeed inhibition of Abl kinase activity by STI-571 results in a dose-dependent decrease in the change in FRET with an IC_{50} of 640 nM under the conditions used (Fig. 3). This result demonstrates the utility of the Rh-CrkΔ-Fl biosensor as a tool for screening for Abl kinase inhibitors.

In summary, we have developed a rapid, non-radioactive Abl kinase assay. The robust fluorescence change of Rh-CrkΔ-Fl upon phosphorylation for the first time allows Abl kinase activity to be monitored in real-time. This biosensor is likely to be useful both as a direct spectroscopic assay for measuring enzymatic catalysis, and, as we have illustrated, for testing Abl kinase inhibitors that might be useful for the treatment of CML. The modular nature of our assembly method for generating Rh-CrkΔ-Fl makes it possible to easily incorporate other small molecule fluorophores or alter the protein kinase substrate for new applications.

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15. The polymerase chain reaction was used to amplify the mouse c-Crk-II sequence (amino acids 2–228) using mutagenic primers encoding the N-terminal sequence (MASDLIEGRC) as well as restriction endonuclease recognition sites for digestion and subsequent ligation into the pTYB3 plasmid (New England Biolabs).
16. Fluorescence measurements were made using a Fluorolog-3 spectrofluorimeter (ISA/Spex) using excitation and emission slit widths of 1.2 and 3 nm, respectively. Reactions (400 uL) containing 50 mM Tris pH 7.6, 2 mM DTT, 10 mM $MgCl_2$, 0.2 mg/mL acetylated BSA, $\sim$60 nM Rh-CrkII-Fl, were initiated by the addition of full-length recombinant Abl kinase[6] (final concentration = $\sim$0.3 nM) in the presence or absence of 5 mM ATP.
17. GFP is a 262 amino acid (29.5 kDa) protein. Moreover, the 5-(*p*-hydroxybenzylidene)-3,5-dihydro-4*H*-imidazol-4-one fluorophore in GFP is buried $\sim$15 Å within the protein.[19]
18. Reactions (200 μL, conditions indicated in ref 16) containing 1.25% DMSO (or STI-571 dissolved in DMSO) were performed in 96-well plates (Corning 00-08-044). Fluorescence measurements were made using a Fluorolog-3 equipped with a Micromax 96-well microtiter plate reader and a 500 nm high pass filter and a 488 band pass filter installed at the excitation (12 nm slit width) and emission (10 nm slit width) slits, respectively.
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