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Design and evaluation of a real-time activity probe for focal adhesion kinase

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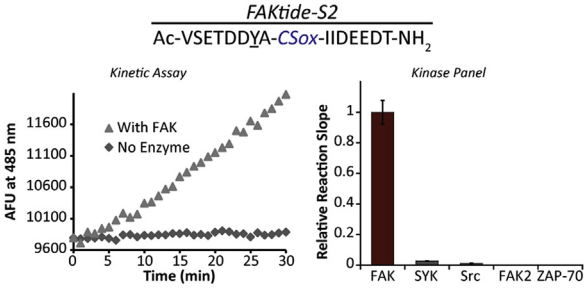
HIGHLIGHTS

- Present a novel direct activity probe for focal adhesion kinase.
- Demonstrate a limit of detection of 1 nM FAK.
- Demonstrate the ability to screen for FAK inhibitors.
- Provide conditions to selectively monitor FAK activity.

GRAPHICAL ABSTRACT



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ABSTRACT

Focal adhesion kinase (FAK) has been identified as a potential therapeutic target for the treatment of metastatic cancers. Herein we describe the design, synthesis and optimization of a direct activity sensor for FAK and its application to screening FAK inhibitors. We find that the position of the sensing moiety, a phosphorylation-sensitive sulfonamido-oxine fluorophore, can dramatically influence the performance of peptide sensors for FAK. Real-time fluorescence activity assays using an optimized sensor construct, termed FAKtide-S2, are highly reproducible ($Z' = 0.91$) and are capable of detecting as little as 1 nM recombinant FAK. Utilizing this robust assay format, we define conditions for the screening of FAK inhibitors and demonstrate the utility of this platform using a set of well-characterized small molecule kinase inhibitors. Additionally, we provide the selectivity profile of FAKtide-S2 among a panel of closely related enzymes, identifying conditions for selectively monitoring FAK activity in the presence of off-target enzymes. In the long term, the chemosensor platform described in this work can be used to identify novel FAK inhibitor scaffolds and potentially assess the efficacy of FAK inhibitors in disease models.

1. Introduction

FAK is a multi-domain non-receptor tyrosine kinase that has been established as a critical signaling node in cell motility [1,2]. Engagement of integrin receptors with extracellular matrix proteins leads to recruitment of FAK and autophosphorylation at Y397 [3]. Subsequent assembly of a multi-protein complex known as a focal adhesion provides a link between the extracellular matrix and

Abbreviations: FAK, focal adhesion kinase; HCC, hepatocellular carcinoma; HTS, high-throughput screening; Sox, sulfonamido-oxine; CSox, cysteine-Sox; DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; K_D, equilibrium dissociation constant.

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the cytoskeleton, allowing for contractile force generation and productive cell movement [4]. Due to the central role of FAK in focal adhesion formation and cell migration, numerous studies have investigated possible links between FAK enzymatic activity and development of metastatic phenotypes. For example, a recent study investigated Y397 phosphorylation on FAK using phospho-specific antibodies in 200 patient-derived hepatocellular carcinoma (HCC) tumors [5]. This work identified a positive correlation between Y397 phosphorylation and tumor staging. Furthermore, siRNA knockdown of FAK expression in HCC cell lines led to inhibition of migration and invasion *in vitro*. These results contribute to the developing body of literature indicating that FAK inhibition could provide a mechanism for the treatment of metastatic tumors [6–9]. We therefore set out to develop a direct activity sensor for FAK that could be used as a tool in high-throughput drug discovery, with the long term goal of investigating the role of FAK enzymatic activity in HCC models.

The classic activity assay for protein kinases involves monitoring the transfer of the γ-phosphoryl group from [γ-^{32}P]ATP to a peptide substrate [10]. This general and sensitive assay format can be applied to virtually any kinase of interest. There are, however, certain limitations associated with this technology. For example, this assay format is inherently discontinuous since reaction quenching and product purification is required prior to data collection. As a result, the real-time analysis of enzymatic function is prohibited. Furthermore, this assay necessitates the use of radioactive material, requiring specialized training and dedicated equipment. Consequently, while radioactivity assays remain the "gold standard" for detailed mechanistic studies of protein kinases, there is a need for the development of assay formats that are compatible with high-throughput screening (HTS) for this important class of drug targets [11,12]. This has led to the development of a variety of assay formats for protein kinases [13]. These assays utilize various mechanisms to monitor kinase activity including production of ADP [14], protease sensitivity of phosphorylated peptides [15], time-resolved FRET [16,17], and the amplified luminescent proximity homogeneous assay format [18]. Although powerful, the broad applicability of these approaches may be limited due to the necessity of specialized instrumentation for data collection. In contrast, fluorescent and luminescent peptide-based assays are capable of providing a real-time, high-throughput readout of kinase activity [19–29]. For example, the Lawrence laboratory has described a robust and generalizable tyrosine kinase assay platform utilizing the environment sensitive nature of the pyrene fluorophore to directly report on phosphorylation of a nearby tyrosine residue [30,31]. Importantly, such assay formats do not require specialized equipment or handling procedures. Consequently, peptide-based direct activity assays are an increasingly desirable option in high-throughput discovery of kinase inhibitors.

In the design of a direct, chemosensor for FAK, we chose to employ the phosphorylation-sensitive sulfonamido-oxine (Sox) fluorophore [32]. Sox is a quinoline derived small molecule fluorophore that can report on the phosphorylation of a proximal amino acid through chelation-enhanced fluorescence induced by Mg^{2+} binding. First generation Sox sensors required deletion of the N- or C-terminal peptide recognition sequence, relative to the site of phosphorylation, in order to pre-organize the peptide for efficient Mg^{2+} binding upon phosphorylation. The Imperiali laboratory has recently described a second generation probe design in which an amino acid in a peptide substrate is replaced with a cysteine derivative of the Sox fluorophore, termed CSox (Fig. 1) [33–36]. CSox-based sensors can be readily accessed through standard Fmoc solid-phase peptide synthesis, allowing for the facile synthesis of direct reporters of kinase enzymatic activity [37], making these probes ideally suited for HTS. Indeed, recent work from the Dalby laboratory has detailed the use of Sox-based sensors to identify eEF-2 inhibitor scaffolds from a 32,960 member small molecule library [38]. Herein, we describe the design, synthesis, and optimization of a CSox-based activity sensor for FAK. We demonstrate the utility of this assay platform for the identification of FAK inhibitors as well as provide conditions for selectively monitoring FAK activity in the presence of off-target enzymes.

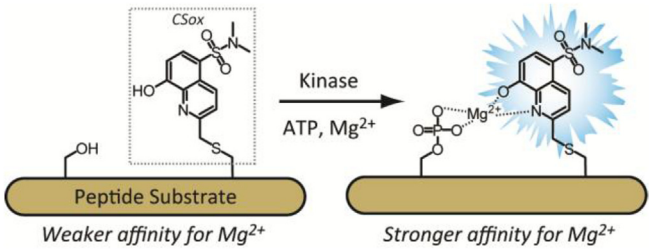

Fig. 1. Direct detection of protein kinase activity using CSox-based chemosensors. A single residue within a peptide substrate of interest is replaced with the CSox unnatural amino acid (left). Phosphorylation is detected through chelation-enhanced fluorescence (right). Phosphate addition can be monitored in real-time by exciting at 360 nm and recording emission at 485 nm.

2. Experimental

2.1. General reagents, instrumentation, and methods

Low metals grade reagents were obtained from Sigma or Alfa Aesar. Fluorescence data were obtained using a BioTek SynergyH1 microplate reader. Assays were excited at 360 nm and emission was monitored at 485 nm at 30 °C. Reactions were performed with 384-well half area plates (Corning, 3824). Total reaction volumes in 384-well plates were 40 μl. Error bars represent the standard deviation of triplicate experiments unless otherwise noted.

2.2. Peptide synthesis

Peptides were synthesized as previously described [33,37] using Fmoc-CSox[TBDPS]-OH and were purified using preparative reverse-phase HPLC (Fig. S1). Peptide identity was verified by ESI-MS (Table S1). Peptide concentrations were determined using the extinction coefficient of Sox, 8247 M^{-1} cm^{-1} at 355 nm, as described previously [32].

2.3. Determination of magnesium K_D

Dissociation constants for Mg^{2+} were determined by measuring the fluorescence of the indicated peptide in the presence of 0.1, 0.5, 1, 5, 10, 50, 100 and 350 mM $MgCl_2$ in a buffer containing 50 mM Tris–HCl (pH = 7.5 at 22 °C) and 150 mM NaCl. Final peptide concentrations were 1 μM. Fluorescence intensities were plotted in Kaleidagraph and fit to a single site binding isotherm.

2.4. Fluorescence intensity dependence on [Mg^{2+}] and [ATP]

Fluorescence intensities of nonphosphorylated and phosphorylated peptides were measured in the presence of the indicated concentrations of Mg^{2+} and ATP. The fold fluorescence increase is the quotient of the fluorescence of the phosphorylated peptide divided by the corresponding nonphosphorylated peptide.

2.5. Kinase assay conditions

Recombinant FAK (Life Technologies, PV3832) was diluted in

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50 mM Tris–HCl (pH = 7.5 at 22 °C), 1 mM EGTA, 0.01% (v/v) Brij, 2 mM DTT, and 10% (v/v) glycerol. Kinase reactions were prepared in 50 mM Tris–HCl (pH = 7.5 at 22 °C), 1 mM EGTA, 2 mM DTT, 0.01% (v/v) Brij, and the optimized concentrations of Mg^{2+} and ATP. Assays were initiated by the addition of enzyme.

The rates of phosphorylation of FAKtide-S1 and FAKtide-S2 were determined by varying the concentration of peptide from 1, 2.5, 5, 10, 20 and 50 μM. Reactions were initiated by the addition of recombinant enzyme. The rate of product formation was determined by correcting for the loss of fluorescence due to substrate turnover, as previously described [32]. The resulting rates were fit to the Michaelis–Menten equation in Kaleidagraph.

Inhibitor assays and assays with closely related enzymes were performed as above in 384-well plates. Inhibitors were purchased from Tocris and closely related enzymes were purchased from Life Technologies. Data were background subtracted using wells without enzyme.

3. Results and discussion

3.1. Sensitivity of FAK to the position of CSox

As a potential substrate we investigated the use of the autophosphorylation site of FAK, Y397 [3]. Using the native sequence (^{391}VSETDDYAEIIDEEDT^{406}), we constructed two sensors: Ac-VSET-*CSox*-DYAEIIDEEDT-NH_2 and Ac-VSETDDYA-*CSox*-IIDEEDT-NH_2 which we term FAKtide-S1 and FAKtide-S2 respectively. These sensor constructs differ in the positioning of CSox (italics), at either the −2 or +2 position relative to the site of phosphorylation (underlined). This spacing between CSox and the site of phosphorylation has previously been determined to provide efficient Mg^{2+} chelation upon phosphorylation [33]. Additionally we synthesized the corresponding phosphorylated peptides (Ac-VSET-*CSox*-DpYAEIIDEEDT-NH_2 and Ac-VSETDDpYA-*CSox*-IIDEEDT-NH_2) as controls, termed FAKtide-P1 and FAKtide-P2 respectively. Since signal generation from CSox-based sensors is dependent upon a change in Mg^{2+} affinity between the nonphosphorylated and phosphorylated states, it is important to determine the affinity of potential substrate peptides and their corresponding phosphorylated controls for Mg^{2+}. The Mg^{2+} K_D values for the FAKtide constructs are shown in Table 1 (see also Fig. S2). These data highlight the need to evaluate the Mg^{2+} affinity of newly synthesized CSox-based probes due to the influence of sequence context on Mg^{2+} affinity [37]. In this case, the relatively tight affinity of these FAK sensors for Mg^{2+} compared to previously described CSox-based chemosensors is likely attributable to the frequency of acidic residues within the FAK autophosphorylation site. Satisfied that each nonphosphorylated peptide showed a reduction in binding affinity to Mg^{2+} compared to the phosphorylated peptide, we investigated the optimal concentrations of both Mg^{2+} and ATP. We screened various combinations of Mg^{2+} and ATP concentrations, using the K_D values of Mg^{2+} as a guide (Table S2). For FAKtide-S1 we found a Mg^{2+} concentration of 1.2 mM and an ATP concentration of 1.0 mM produced a robust 5.0-fold increase in fluorescence between the phosphorylated peptide and the substrate peptide (Table 1). While for FAKtide-S2 a 4.3-fold increase was observed at a Mg^{2+} concentration of 1.4 mM and an ATP concentration of 1.0 mM (Table 1).

Having identified optimal concentrations of ATP and Mg^{2+}, we proceeded to determine if FAKtide-S1 and FAKtide-S2 could be used as substrates by FAK in an *in vitro* assay. Kinase reactions with FAKtide-S1 showed relatively little activity when assayed with recombinant FAK as compared to analogous reactions with FAKtide-S2 (Fig. 2). However, we were unable to saturate the enzyme with either substrate in order to accurately determine kinetic constants

Table 1
Mg^{2+} affinities and fold fluorescence increases for FAKtide-S1 and FAKtide-S2 constructs.

Peptide	Mg^{2+} K_D (mM)	Fold fluorescence increase[a]
FAKtide-S1	11.9 ± 2.3	
FAKtide-P1	1.2 ± 0.4	5.0 ± 0.2
FAKtide-S2	10.0 ± 1.2	
FAKtide-P2	1.4 ± 0.3	4.3 ± 0.1

[a] Fold fluorescence increases were determined by dividing the fluorescence of the phosphorylated peptide by the nonphosphorylated peptide. Assays were conducted in the presence of 1.0 mM ATP, Mg^{2+} concentrations were set at the K_D of the corresponding phosphorylated peptide in each set.

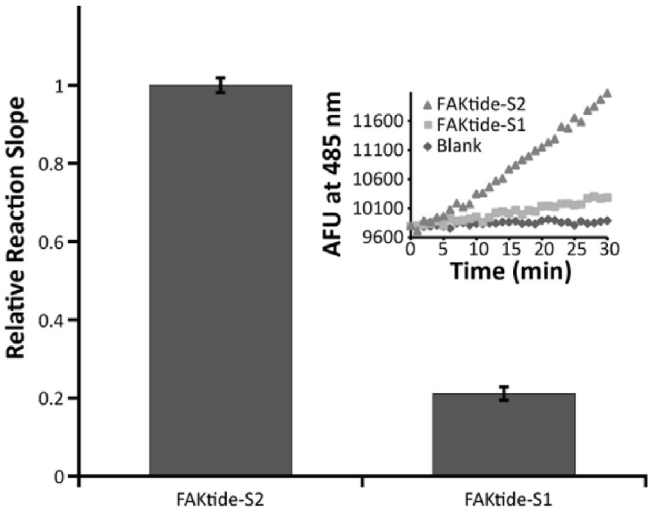

Fig. 2. Direct comparison of CSox-based chemosensors for FAK demonstrates that FAKtide-S2 is a more efficient probe for FAK activity. Activity assays were conducted using the optimal concentrations of Mg^{2+} for each sensor. Reactions contained equal concentrations of each sensor (10 μM) and were initiated with 5 nM FAK. Reaction slopes are scaled relative to the activity of FAKtide-S2. The inset shows the increases in fluorescence with time for each experiment.

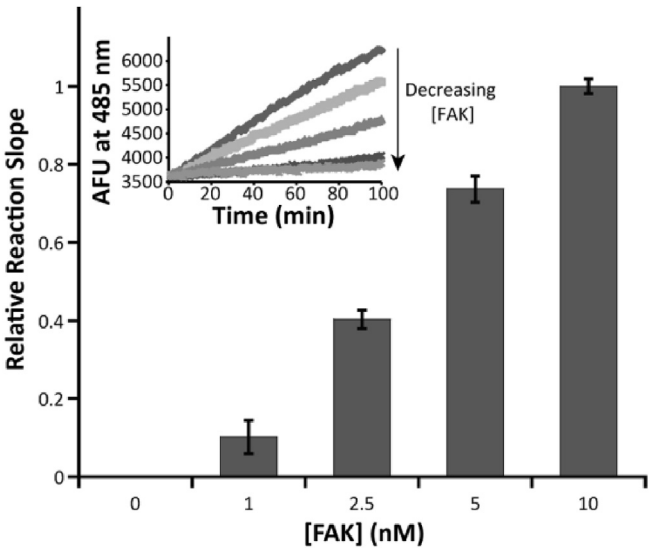

Fig. 3. FAKtide-S2 is capable of reproducibility detecting FAK concentrations as low as 1 nM. Reactions containing FAKtide-S2 (1 μM) were initiated with the indicated concentration of FAK. Reaction slopes are scaled relative to the activity of 10 nM FAK. The inset shows the increases in fluorescence with time for each experiment.

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(Fig. S3). Nonetheless, from these results we inferred that the position of CSox in FAKtide-S1 may interfere with binding and/or catalysis. These results are not surprising when compared with general trends in substrate specificity of tyrosine kinases. In determining the substrate specificity of a variety of both receptor and non-receptor tyrosine kinases Cantley and colleagues demonstrated the almost universal occurrence of acidic amino acid residues at the −2 position relative to the site of phosphorylation [39]. Furthermore, it was shown that at the +2 position a larger variety of residues were accepted including branched-chain as well as phenylalanine residues thus suggesting a higher tolerance among tyrosine kinases for bulky amino acids at this site. Encouraged by these results, we decided to proceed with assay optimization using FAKtide-S2.

3.2. FAK limit of detection with FAKtide-S2

Recombinant enzyme can represent a significant cost in developing high-throughput assays. Consequently, the ability of the sensor to report on low nanomolar levels of FAK would further its utility in HTS campaigns. In order to test this parameter with FAKtide-S2, we determined the limit of detection for recombinant FAK under optimized conditions. We found that FAKtide-S2 is able to faithfully report on FAK activity at concentrations as low as 1 nM (Fig. 3). This detection limit is within the range of previously described CSox-based activity probes [28,29,35] as well as other commonly used high-throughput methodologies for detecting protein kinase activity [40–42]. Furthermore, analysis of this data at 2.5 nM, 5 nM and 10 nM FAK showed that this assay is highly

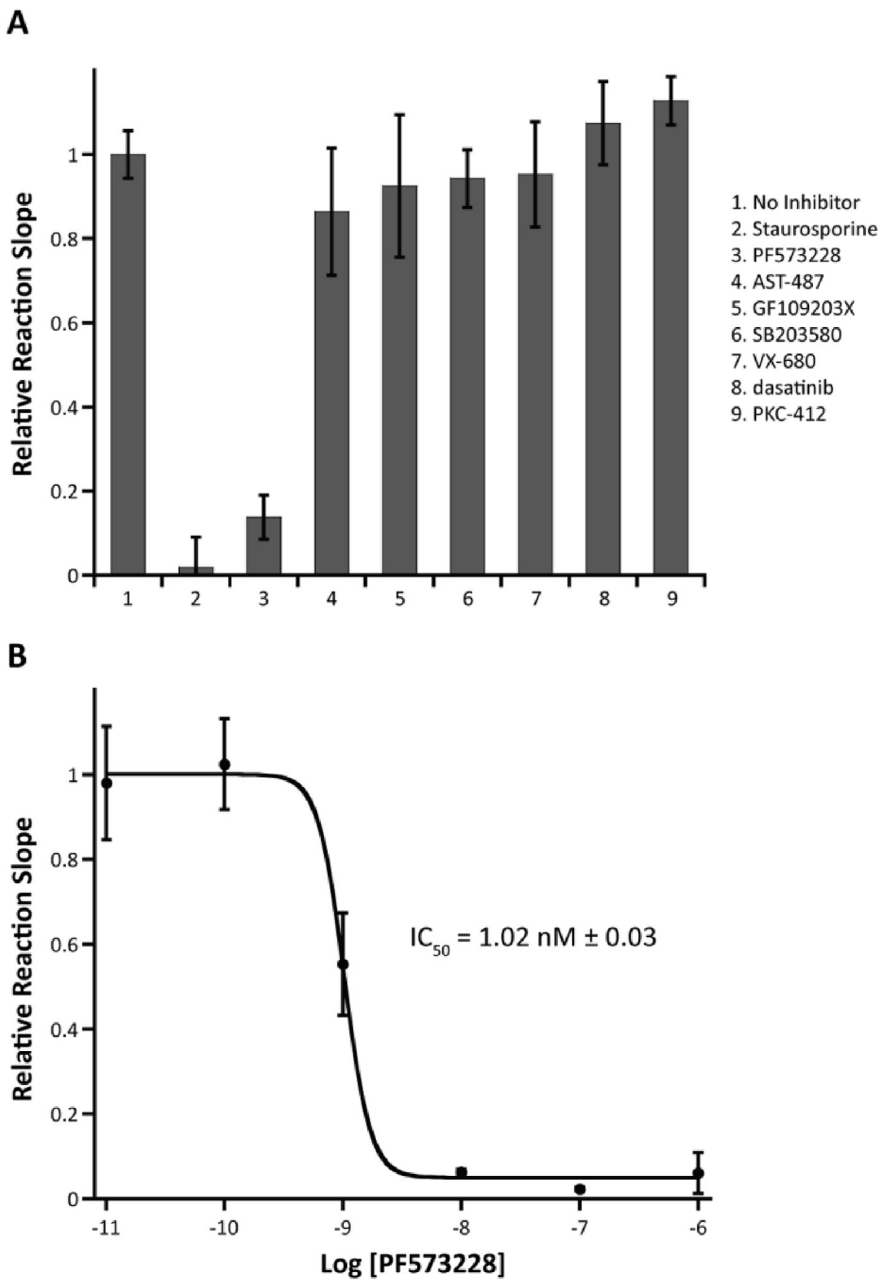

Fig. 4. FAKtide-S2 can be utilized in a high-throughput format to identify FAK inhibitors. (A) Assays contained 2.5 nM FAK and 1 μM of the indicated inhibitor. Potent inhibition is observed for both stauropsorine and PF573228. (B) FAK activity (5 nM) as a function of increasing PF573228 concentration. All assays were conducted in 384-well plates with 1 μM FAKtide-S2. In each panel, reaction slopes have been scaled relative to the activity of an uninhibited assay.

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reproducible, with respective Z'-factors [43] of 0.72, 0.81 and 0.91. These data indicate the potential of FAKtide-S2 for HTS.

3.3. FAKtide-S2 as a tool for FAK inhibitor discovery

Building on these results, we transitioned the FAKtide-S2 assay into a high-throughput 384-well plate format and assembled a small library of kinase inhibitors with known affinities for FAK in order to validate our screening platform. To find control compounds with no known affinity for FAK, we utilized an existing profile of the binding affinities of 72 small molecules across 442 kinases [44]. This analysis identified six potential negative control compounds (AST-487, GF109203X, SB203580, VX-680, dasatinib and PKC-412) that were reported to have K_Ds of >10 μM for FAK. As positive controls, we included the highly promiscuous kinase inhibitor staurosporine [44] as well as the well-characterized FAK inhibitor PF573228 [45]. To demonstrate the utility of FAKtide-S2 for HTS, we interrogated each member of the compound library, at a concentration of 1 μM, against recombinant FAK using FAKtidie-S2 to directly report on the inhibition of FAK. The results of this experiment (Fig. 4A) validate staurosporine and PF573228 as relatively potent inhibitors of FAK. Furthermore, PKC-412, VX-680, dasatinib, AST-487, SB203580 and GF109203X showed little to no inhibition of FAK at 1 μM in accordance with literature data [44]. Therefore, this pilot screen shows that FAKtide-S2 can be used as a tool in HTS of inhibitors for FAK in order to identify lead compounds. In addition, the kinetic readout of this assay format minimizes false positive sensor signals observed in endpoint assays due to autofluorescence of inhibitor compounds. An additional benefit of this assay platform is the ability to rapidly determine inhibitor potency. Using FAKtide-S2 we were able to determine the IC_{50} of PF573228 for FAK under optimized assay conditions (Fig. 4B). The observed IC_{50} correlates with literature values [45] and indicates that the same assay format can be used to investigate the structure-activity relationships of hits identified from HTS.

3.4. Selective monitoring of FAK activity using FAKtide-S2

In the long term, our laboratory seeks to utilize CSox-based activity sensors to define FAK activity perturbations in disease states such as HCC [5]. Such data could be used to establish a direct biochemical link between alterations in FAK activity and disease phenotypes. Moreover, a selective FAK activity sensor could be used to monitor FAK inhibitor efficacy in disease models. As an initial step towards this goal, we interrogated the selectivity of FAKtide-S2 across a panel of closely related protein kinases including SYK, Src, FAK2, and ZAP-70 [46]. Within this panel of kinases we observed significant off-target activity from SYK (59%) (Fig. 5A). These data indicate that FAKtide-S2 could be utilized for SYK inhibitor screening as well as to assess the selectivity of FAK inhibitors. However, we sought to further increase the selectivity of the assay by employing promiscuous inhibitors to suppress off-target activity. During the optimization of the FAKtide-S2 assay for HTS we identified PKC-412 as a small molecule scaffold with no appreciable effect on FAK activity at concentrations up to 1 μM (Fig. 4A). Interestingly, the selectivity profile of PKC-412 indicated broad spectrum inhibition across the kinome and, in particular, for SYK [44]. Hence, we hypothesized that at appropriate concentrations PKC-412 could be employed to suppress off-target kinase activity on FAKtide-S2 while preserving signal from FAK in the assay. Accordingly, we investigated the ability of PKC-412 to suppress off-target activity in the kinase panel. Indeed, the addition of PKC-412 reduced off-target activity to background levels, allowing for the selective monitoring of FAK activity (Fig. 5B). With further optimization it may be possible to develop a FAKtide-S2 assay that could be used to study FAK biology in complex biological samples such as cell lysates or tissue homogenates [37,47–49]. However, it is important to recognize that FAKtide-S2 could be utilized as a substrate by tyrosine kinases not present in the current panel. Thus, validation of this assay in heterogeneous biological samples will require a more detailed analysis of probe specificity in cell lysates [37].

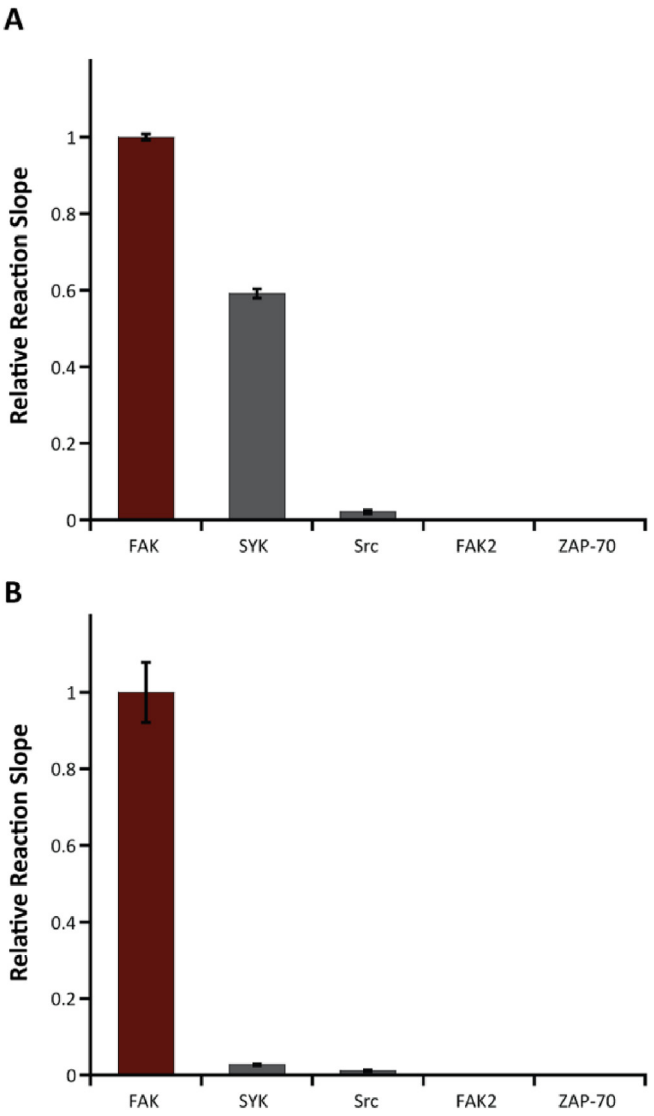

Fig. 5. Suppression of off-target activity using 10 μM PKC-412 allows for the selective monitoring of FAK activity using FAKtide-S2 (1 μM). (A) Significant off-target activity is observed from SYK (59%). (B) FAK activity can be resolved by employing the promiscuous kinase inhibitor PKC-412. Reactions were initiated with 5 nM of the indicated enzyme. Reaction slopes in both panels have been scaled to the activity of FAK.

4. Conclusions

We have described the development and optimization of a chemosensor for FAK. The sensor constructs indicated a preference for the CSox amino acid at the +2 position, relative to the site of phosphorylation, within the FAK autophosphorylation site. The optimal construct, FAKtide-S2 is an efficient sensor capable of detecting FAK concentrations as low as 1.0 nM. Moreover, the reproducibility of this assay platform (Z' = 0.91) provides a robust

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mechanism for HTS of potential FAK inhibitors. In support of this application, we have miniaturized this assay to a 384-well plate format and have demonstrated the ability to screen small molecules for FAK inhibition. Lastly, we define the selectivity profile of FAKtide-S2 and provide conditions for monitoring FAK activity in the presence of off-target enzymes. Together, this work provides a reliable platform for identification and characterization of FAK inhibitors. In the long term, this novel chemosensor may find applications in probing FAK biochemistry in human disease states, such as HCC, as well as monitoring inhibitor efficacy in disease models.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2015.09.025.

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