Published in final edited form as:

J Am Chem Soc. 2010 August 25; 132(33): 11727–11735. doi:10.1021/ja104491h.

A Coiled-Coil Enabled Split-Luciferase Three-Hybrid System: Applied Toward Profiling Inhibitors of Protein Kinases

Benjamin W. Jester, Kurt J. Cox, Alicia Gaj, Carolyn D. Shomin, Jason R. Porter, and Indraneel Ghosh*

Department of Chemistry and Biochemistry, University of Arizona, 1306 E. University Blvd., Tucson, AZ 85721

Abstract

The 518 protein kinases encoded in the human genome are exquisitely regulated and their aberrant function(s) are often associated with human disease. Thus, in order to advance therapeutics and to probe signal transduction cascades there is considerable interest in the development of inhibitors that can selectively target protein kinases. However, identifying specific compounds against such a large array of protein kinases is difficult to routinely achieve utilizing traditional activity assays, where purified protein kinases are necessary. Toward a simple, rapid, and practical method for identifying specific inhibitors, we describe the development and application of a split-protein methodology utilizing a coiled-coil assisted three-hybrid system. In this approach, a protein kinase of interest is attached to the C-terminal fragment of split-firefly luciferase and the coiled-coil Fos, which is specific for the coiled-coil Jun, is attached to the N-terminal fragment. Upon addition of Jun conjugated to a pan-kinase inhibitor such as staurosporine, a three-hybrid complex is established with concomitant reassembly of the split-luciferase enzyme. An inhibitor can be potentially identified by the commensurate loss in split-luciferase activity by displacement of the modified staurosporine. We demonstrate that this new three-hybrid approach is potentially general by testing protein kinases from the different kinase families. To interrogate whether this method allows for screening inhibitors, we tested six different protein kinases against a library of 80 known protein kinase inhibitors. Finally, we demonstrate that this three-hybrid system can potentially provide a rapid method for structure/function analysis as well as aid in the identification of allosteric inhibitors.

Introduction

Protein kinases catalyze the transfer of the γ -phosphate of ATP to specific serine, threonine or tyrosine residues on a protein substrate. Protein kinases play a central role in almost all cellular signaling cascades and are tightly controlled both spatially and temporally. The aberrant function of many protein kinases has been linked to numerous diseases, namely cancer,^{1,2} inflammation,^{3,4} and metabolic disorders.⁵ After intense efforts, protein kinases have emerged as an important class of biological targets amenable to small molecule intervention, resulting in both therapeutics and probes for interrogating signal transduction.⁶ Several inhibitors, such as Imatinib (Gleevec),⁷ have emerged as FDA-approved therapeutics with many more in clinical development.^{2,8} Typically, protein kinase inhibitors function by outcompeting ATP at the architecturally conserved active site, which poses a significant hurdle as the approximately 518 human protein kinases⁹ display considerable

*To whom correspondence should be addressed: Professor Indraneel Ghosh, The University of Arizona, Department of Chemistry & Biochemistry, 1306 E. University Blvd., Tucson, AZ 85721, Phone: 520-621-6354, Fax: 520-621-8407, ghosh@email.arizona.edu.

Supporting Information Available. Experimental details, chemical structures and library screening results, and the complete citations for refs 10, 11 and 21. This material is available free of charge via the Internet at <http://pubs.acs.org>.

sequence and structural conservation. Recent studies have shown that numerous FDA approved kinase inhibitors, though effectively inhibiting their intended targets exhibit limited selectivity when tested against a large panel of protein kinases.^{8,10,11} This often unintended promiscuity or polypharmacology displayed by kinase inhibitors can be potentially beneficial by targeting several protein kinases, such as the broad spectrum activity of PKC-412, an analog of the pan-kinase inhibitor staurosporine, or the activity against c-KIT and PDGFR exhibited by Imatinib. Although promiscuity has both potential benefits and pitfalls in therapeutics, it is clearly a significant liability for the selective elucidation of the role of a specific kinase in signal transduction.¹² Shokat and co-workers have provided an important rationale for the need for protein kinase selective small molecule probes,^{13,14} since they function at a significantly different temporal scale than biological knockdowns and thus more accurately reflect the cellular consequences of small molecule therapeutics.

Not surprisingly, there has been much recent effort in profiling protein kinases against small molecule targets.¹⁵ The gold standard remains the direct measurement of enzymatic activity as exemplified by profiling studies from Cohen and co-workers,^{12,16-18} however this usually requires access to a large number of purified and functional protein kinases and radioactive based assays for sensitivity. More recently, Fabian and co-workers have described a kinase inhibitor profiling strategy that does not require the expression and purification of each protein kinase and is based upon the ability to rapidly express kinases on the surface of phage that can be subsequently interrogated for small molecule binding through competition experiments.^{10,11} In related approaches that do not require purified protein kinases, chemical inducers of dimerization (CIDs)¹⁹ have been employed for addressing protein kinase inhibitor selectivity in a cellular context.^{20,21} These CIDs stemming from the seminal work by Schreiber and co-workers^{22,23} were utilized by Liu and co-worker in a yeast three-hybrid approach enabled by a dexamethasone–FK506 CID,²⁴ while more recently, Cornish and co-workers established an elegant dexamethasone–methotrexate based CID for three-hybrid and related applications.²⁵ What is common among the CID approaches is the availability of a high affinity small molecule ligand and a protein receptor pair of considerable size to impart affinity and selectivity. For example, the FK506 binding protein that binds FK506 is the smallest at 12 kD,²⁴ dihydrofolate reductase that binds methotrexate is 18 kD,²⁵ the glucocorticoid receptor that binds dexamethasone is 31 kD,²⁵ and the estrogen receptor that binds estradiol is 29 kD.²⁶ Building on the above observations, we envisioned an easily implemented, modular, and general three-hybrid kinase inhibitor screen utilizing a new peptide based CID that can function in cell free translation systems.^{27,28} Towards this goal, we detail our progress towards the design, validation, and application of a new three-hybrid design paradigm, where one pair of a coiled-coil peptide conjugated to a kinase ligand,²⁸ can potentially function as a CID and bridge a user-defined kinase attached to one split-protein fragment with the cognate coiled-coil peptide attached to the second fragment (Figure 1). This three-hybrid approach would potentially provide a new synthetically accessible CID and complement the large protein receptor/ligand pair CID approaches for profiling protein kinases in an *in vivo* context. The peptide-small molecule CID would be particularly suited for lysate based translation systems or functions in an extracellular context, where cell permeability is not a hurdle.²⁷

The proposed three-hybrid approach for designing a protein kinase inhibitor sensor relies both on split-protein design as well as the specificity of natural and designed heterodimeric coiled-coils. Split-protein reassembly or protein fragment complementation methods rely on the interaction driven conditional reassembly of carefully fragmented proteins.²⁹ Beginning with split-ubiquitin,³⁰ which has been recently utilized in a CID approach,³¹ several new split-protein sensors that generate an easily detectable output have been developed, such as GFP,³²⁻³⁴ dihydrofolate reductase,³⁵ β -lactamase,³⁶⁻³⁸ firefly luciferase,^{27,39} and *Gaussia*

Luciferase.⁴⁰ Similarly, natural and designed coiled-coils have emerged as one of the smallest synthetically accessible self-assembling modular units with wide utility in synthetic biology.⁴¹⁻⁴⁴ To the best of our knowledge, coiled-coil enabled three-hybrid systems have not been utilized as a means for driving the reassembly of split-proteins to provide a direct read-out for the binding of a ligand to a protein kinase and its subsequent displacement by an inhibitor (Figure 1). We envisioned that our general biosensor architecture for interrogating protein kinase-ligand binding would comprise the fragmented firefly luciferase, with one fragment attached to a protein kinase of interest and the complementary fragment attached to the Fos coiled-coil peptide (Figure 2A). Upon the addition of the cognate Jun coiled-coil conjugated to the pan kinase inhibitor, staurosporine (**1**), the split-protein reporter fragments would be brought into proximity to allow for split-protein reassembly and restore the activity of the reporter (Figure 2A). We envisioned that the addition of a staurosporine competitive small molecule would result in the displacement of Jun-staurosporine (**2**) and thereby dissociate the luciferase fragments with corresponding loss in luminescence.

Herein, we successfully demonstrate the feasibility of our new coiled-coil enabled CID that allows for the construction of a three-hybrid biosensor, utilizing the reassembly and subsequent activity of split-firefly luciferase as a sensitive luminescent read out. We utilize an *in vitro* translation system to test our methodology,²⁷ which provides a rapid assay platform with *in situ* production of the necessary proteins without the need for purification, a potential impediment for large scale experiments.⁴⁵ Moreover, the study of protein kinases and their inhibitors in a lysate environment that perhaps approximates a complex *in vivo* setting may be beneficial for eliminating generic off-target hits.^{45,46} To demonstrate feasibility we began with the well-studied cAMP dependent protein kinase A, catalytic subunit α (PKA). To test generality, eleven additional protein kinases that span the kinome were tested successfully, and six were subsequently tested against a panel of 80 known inhibitors. Further studies were designed to test if the three-hybrid methodology can be potentially utilized for interrogating the importance of specific residues implicated in protein kinase activity and in identifying allosteric inhibitors which do not directly target the active site.

Results and Discussion

Demonstration of Ternary (Three-hybrid) Complex Assembly and Inhibition

PKA was selected as an initial test protein kinase based on our previous experience with PKA, where we had established that it effectively binds a Jun-staurosporine conjugate.^{28,47} The catalytic domain of PKA was conjugated to the C-terminal fragment of luciferase, Cfluc, through a 13-residue linker (Cfluc-PKA), while the Fos peptide was conjugated to the N-terminal fragment of luciferase, Nfluc (Fos-Nfluc). The addition of Jun-staurosporine (**2**) was expected to lead to the formation of a ternary complex containing both proteins and CID (Figure 2A). Concomitant expression of both mRNA constructs in reticulocyte lysate exhibited little luminescence, however upon addition of **2**, the signal increased by >100-fold (Figure 2B). To validate that the observed signal was indeed due to the CID, either free Jun peptide or **1** modified with three β -alanine residues (St3 β A, **3**), to replicate the linker between **1** and Jun, were added to Cfluc-PKA and Fos-Nfluc. The negligible signal observed for all the components, excepting **2**, suggests that background luciferase reassembly was minimal unless the full CID was present (Figure 2B). Next, we wanted to test the optimal orientations of the attached PKA and Fos to the luciferase fragments. Thus, Fos and PKA were attached to the opposite halves of luciferase, creating Cfluc-Fos and PKA-Nfluc respectively. This new orientation was tested for signal enhancement upon addition of **2** (Figure 2C) and exhibited a >10-fold increase in luminescence upon the addition of CID, considerably less than the >100 fold signal enhancement in the alternate orientation.

The utility of this split-protein three-hybrid approach assay ultimately lies in its potential as a methodology for measuring the selectivity of a protein kinase for its inhibitors. Thus, in order to determine if we could measure protein kinase inhibition, the ternary complex was titrated with staurosporine (**1**) (Figure 2D). The observed concentration-dependent loss in luminescence follows a characteristic sigmoidal curve, with an IC_{50} of 93 nM under our experimental conditions. The measured IC_{50} value is 5-fold higher than the reported dissociation constant, K_d , of 19 nM recently determined by Karaman and co-workers¹¹ and is likely a function of the concentration of **2**, 120 nM, as well as the potential affinity between the split-luciferase halves. The ability to inhibit the reassembled luciferase is important because similar experiments with split-GFP are difficult, as the reassembled complex has been observed to be kinetically trapped.⁴⁸ Importantly, this set of proof of principle experiments validated that ligand binding capable protein kinase domains could be produced *in situ* and that the reassembly of split-luciferase, brought about by the coiled-coil enabled CID, could be reversed by addition of a small molecule ligand for the kinase. Furthermore, testing the different orientations of the protein kinase with respect to the luciferase fragments clearly demonstrated a significant signal-to-noise advantage afforded by the Cfluc-PKA/Fos-Nfluc, leading to all subsequent protein kinases being tested as Cfluc conjugates.

Adaptability to Diverse Protein Kinases

While our three-hybrid approach was successful with PKA, we next sought to determine if it could be applied to other protein kinases known to bind the pan-kinase inhibitor, staurosporine. The human kinome is typically segregated into seven genetically and functionally diverse groups⁹ (Figure 3A) and at least one kinase from each, except the CK1 group, was selected for further validation, and their appropriate catalytic domains were cloned in place of PKA in our system (Supplementary Information, Table S1).

The respective mRNAs for ten different protein kinases (PKA, Akt1, Akt2, PKC α , PDGFR, FGFR1, MAP3K11, SLK, PIM1, and CLK2) fused to Cfluc were expressed in reticulocyte lysate and tested for luciferase reassembly and concomitant luminescence only upon addition of **2**. All ten tested in the three-hybrid system showed significant signal enhancement upon addition of **2** (Figure 3C). Thus, this experiment demonstrated that this new three-hybrid approach is potentially general across the kinome, when a potential protein kinase ligand can be identified that retains binding affinity upon conjugation to Jun. Having established successful reassembly of split-luciferase with the ten protein kinases upon addition of **2**, we then determined if the addition of PKC-412 (**4**), a better mimic of the staurosporine conjugate,⁴⁷ could reverse luciferase reassembly and knock down signal. Either no inhibitor (vehicle only) or **4** at a concentration of 10 μ M was added to the lysate to measure signal loss (Figure 3C). In each case, **4** caused a >50% loss in luminescence relative to no inhibitor, demonstrating that the CID-dependent complex formation can be effectively disrupted upon addition of a competitive inhibitor for a variety of protein kinases.

Inhibitor Profiling of Several Kinases

Next we turned to establishing whether this three-hybrid assay could be effectively utilized for testing a large number of inhibitors against several of the protein kinases that we had demonstrated provided functional three-hybrid assemblies and could be subsequently inhibited by the addition of a competitive ligand (Figure 3). Towards this goal, PKA, AKT1, FGFR1/FLT2, and PIM1 were each tested for inhibition by 80 known kinase inhibitors from a commercial kinase panel, the Tocriscreen Kinase Inhibitor Toolbox, with some substitutions (Supplementary Information, Table S2). Each Cfluc-kinase fusion was translated along with Fos-Nfluc, followed by the addition of **2** to induce ternary complex formation and aliquoted into a 96-well plate containing a unique inhibitor in each well. The

small molecule inhibitors were present at a final concentration of 10 μ M in the lysate and incubated for 1 hour to allow for competitive displacement of **2**. Positive controls, containing **2**, and negative controls, containing neither **2** nor inhibitor, were also included on each plate. Plates were prepared in duplicate for each kinase, and inhibitor potency was determined as percent inhibition relative to vehicle alone. To ensure small molecules were targeting the desired staurosporine/kinase interaction, a Fos-Nfluc and Cfluc-Jun heterodimer²⁷ was used as a control for identifying potential inhibitors of either luciferase⁴⁹ or the Fos/Jun coiled-coil itself. Any loss in signal observed upon addition of inhibitor to Fos-Nfluc and Cfluc-Jun was subtracted from the kinase percent inhibition values (Supplementary Information, Table S5).

All four kinases used in this proof-of-principle three-hybrid screen showed very distinct inhibitory profiles against the panel of kinase inhibitors (Figure 4A-D), with AKT1 being targeted by very few inhibitors from the panel. A cursory examination of the complete data set (Supplementary Information, Table S2) clearly reveals that this three-hybrid method can be potentially utilized to predict inhibitor selectivities. For example, the positive inhibitor control, PKC-412 (**4**), inhibited all four kinases with inhibition values between 55% and 73%. Similarly, the known PKA inhibitor H89 (**5**) was seen to inhibit PKA and AKT1, at 67% and 21% inhibition respectively, but showed no appreciable inhibition of the other two protein kinases, consistent with previous reports.¹⁸ A group of structurally similar CDK inhibitors, aminopurvalanol A (**6**), purvalanol A (**7**), and purvalanol B (**8**), were found to inhibit FGFR1 at 68%, 28%, and 58% respectively. Another group of inhibitors (PI 828 (**9**), LY 294002 (**10**), and NU 7026 (**11**)) sharing structural similarity and originally designed to target members of the phosphoinositide 3-kinase family, all selectively inhibited PIM1 between 37% to 78% while not significantly inhibiting the other three kinases. Given that **10** has been recently co-crystallized with PIM1 in a crystallization screen,⁵⁰ it is perhaps not surprising that both **9** and **11** are also inhibitory. We also observed the lack of activity in this assay for an inhibitor reported to be selective for AKT1, API-2 (**12**), which has been shown to prevent the activation of AKT1 *in vivo*, by prohibiting its localization to the inner-membrane surface, which requires the Pleckstrin homology (PH) domain.^{51,52} It is possible that the reported activity exhibited by this small molecule against AKT1 has been abolished, as the Cfluc-AKT1 fusion used in the three-hybrid system contains only the protein kinase domain and lacks the PH domain.

To further interrogate the ability of this three-hybrid system to potentially profile inhibitors of protein kinases that exist as oligomers in their native state, we turned towards testing the catalytic domain of the AMP-activated protein kinases, AMPK1 and AMPK2. We found that either Cfluc-AMPK1 or Cfluc-AMPK2 in the presence of Fos-Nfluc and **2** provided signals of >100-fold over background luminescence. This demonstrated that the catalytic domain for a natively trimeric kinase^{53,54} can potentially be utilized independently from the oligomerization domain in this three hybrid system. Subsequently, both kinases were tested against the 80 compound panel and the results are summarized in Supplementary Table S2.

With profiling results of the panel of 80 known kinase inhibitors against six kinases in hand, we turned towards comparing the results from this study with published large-scale profiling studies.¹⁰⁻¹² Of the 80 inhibitors tested in our panel, 9 have been previously tested utilizing the phage-display capture methodology^{10,11} and 17 have been recently profiled in standard kinase assays by Cohen and co-workers.¹² The selectivity profiles in our three-hybrid approach were found to mirror the previously measured selectivities reported utilizing phage-display capture (Supporting Information, Table S3). Similarly, the compound selectivities utilizing this methodology were found to be similar to that utilizing activity based assays for 13 compounds. Of the set of 4 compounds tested between all three methods there was a single discrepancy, where compound SP 600125 demonstrated no inhibition

against PIM1 in both our experiments and the phage-display method,¹⁰ while the activity based assay showed 60% inhibition.¹² Noteworthy differences were also found for 3 compounds tested in the traditional assay for PIM1 and AMPK1 (Supporting Information, Table S4), where significantly more inhibition was observed compared to our method, however corresponding data from the phage-display approach have not been reported for these compounds.¹⁰ A major difference between the activity based assay and this methodology is the relative ATP concentrations, where the activity based methods utilize between 5-50 μ M,¹² while this method utilizes cellular levels of ATP (0.5 – 1 mM),⁵⁵ which may result in different levels of inhibition observed for ATP competitive compounds. We plan to systematically investigate these potential differences between methodologies in future experiments.

Overall these experiments clearly demonstrate that this three-hybrid assay provides a potentially simple method for rapidly profiling *in situ* expressed kinases against a large set of inhibitors. Even with this test set of 80 known kinase inhibitors, many of which are reported as selective inhibitors and have been utilized in probing biology (Supplementary Information, Table S2), it is clear that profiling compounds against several protein kinases can reveal new activities against off target kinases. Moreover, this set of experiments also speaks to three-hybrid design considerations, such as the need for testing the three-hybrid system upon inclusion of additional domains in the case of AKT1.

Effect of Kinase Activation on Inhibitor Binding

Having established that the three-hybrid assay can be utilized for profiling inhibitors against particular kinases, we next wanted to test whether this methodology would allow us to study the protein kinase domain as a function of its ligand binding properties. Protein kinases typically require one or more specific serine, threonine, or tyrosine residue to be phosphorylated in order to achieve a fully active conformation, and this modification may also be important for ATP and inhibitor binding at the kinase active site.^{56,57} This can occur through either cis-⁵⁸ or trans-phosphorylation⁵⁹ events, with phosphorylation of residues adjacent to the active site being particularly critical.⁶⁰⁻⁶² Reticulocyte lysate is known to contain endogenous protein kinase activity^{63,64} that could potentially result in the phosphorylation of protein kinases attached to the luciferase fragments. However it is unclear whether the protein kinases in the three-hybrid assay are present in an active phosphorylated form. This ambiguity regarding phosphorylation is also the case when protein kinases are expressed in *E. coli* or yeast as in the previously reported high throughput methods for protein kinase profiling.^{10,21} Thus we set out to test the importance of known residues that potentially play a role in protein kinase activation and possibly ligand binding. To this end, mutations were introduced in both PAK1 and AKT2 that replaced particular serine and threonine residues with aspartate or glutamate to chemically mimic the phosphorylated, active state or alternatively to alanine to potentially mimic the dephosphorylated, inactive form. In the case of PAK1, mutation of a so-called T-loop residue (T423) to glutamate^{65,66} demonstrated a clear increase in luminescence of the CID induced complex over either the wild-type or the alanine mutant (Figure 5A). These results to a first approximation suggest that PAK1 is likely not in a fully active conformation in the lysate and an unphosphorylated threonine prevents it from binding to the modified staurosporine. It is also interesting, that the modified staurosporine can still bind PAK1 with a T423A substitution, which suggests that the active site is still capable of ligand binding. Next we tested this approach on AKT2, which has been reported to be primarily activated by phosphorylation at T309 and S474.⁶⁷ In addition to phosphorylation of the T-loop, AGC kinases are known to also require phosphorylation at the hydrophobic motif for optimal activation.⁶⁸⁻⁷⁰ Mutating both of these residues to aspartate, only increased the luminescence by 2-fold over the wild-type, suggesting that AKT2 may be partially

phosphorylated in the lysate (Figure 5B). Surprisingly, mutating both of these residues to alanine, thereby eliminating the possibility of phosphorylation at either site, causes only a small loss in binding to the CID (Figure 5B). Overall, these results with AKT2 suggest that the (T309A, S474A) mutant, that has been proposed to mimic the inactive form and exhibits a nearly 40-fold increase in luminescence upon addition of **2**, is still very competent for binding active-site targeted inhibitors. Thus these results demonstrate that systematic mutagenesis studies of protein kinases in the context of this three-hybrid system may allow for understanding the determinants that govern inhibitor binding and may find use in delineating the consequences of protein kinase mutations that result in drug resistance.⁷¹

Allosteric Inhibition of AKT1

Having demonstrated that the three-hybrid method has the potential to monitor potential conformational changes that occlude ligand binding at the active site, we turned to investigating the utility of this method for testing allosteric inhibitors that act at sites distal to the active site. Allosteric inhibitors can act by inducing an inactive conformation upon binding (Figure 6A). The AKT1 and AKT2 inhibitor Akt1/2 (**13**) is particularly interesting as it has been previously shown to only impact the activity of a protein kinase constructs containing both the PH domain and the stretch of residues connecting it to the kinase domain.^{72,73} The proposed mechanism of action suggests that binding of this inhibitor induces a conformation which prevents substrate access to the AKT1 active site.⁷⁴ Thus, we set out to compare the effectiveness of **13** against the protein kinase domain only, as well as the full-length AKT1 construct (Figure 6C). We also wanted to test PKC-412 (**4**), known to target the active site, against both constructs. Finally we wished to interrogate the reported allosteric AKT1 inhibitor **12**, which did not display significant inhibition against the kinase domain alone (Figure 4). For Akt1/2 (**13**) we observed a >70% inhibition of the full-length kinase while not inhibiting the kinase domain alone (Figure 6D), while PKC-412 (**4**) targeted both domains equally. Additionally, the allosteric AKT1 inhibitor **12** showed a similar effect for both forms of AKT1, suggesting that this method of analysis will not allow for identification of inhibitors that do not necessarily block the active site but function by other mechanisms such as preventing subcellular translocation.^{52,75} These studies in concert demonstrate that simultaneously screening varying isoforms of a protein kinase in the three-hybrid assay provides us with a simple method to identify selective allosteric inhibitors that can block the active site such as **13**.

Conclusions

As the role of protein kinases continue to be unraveled in a wide array of signal transduction cascades and diseases, the need for new methodologies capable of rapidly probing the more than 500 human protein kinases for inhibitor selectivity has become increasingly evident. Simple and rapid approaches that are amenable for large-scale screens would potentially aid in the discovery of new ligands as reagents for probing biology as well as early candidates in the quest for a drug. Toward this long-term goal we have demonstrated a new three-hybrid paradigm, where a kinase inhibitor conjugated to one pair of a coiled-coil serves as a chemical inducer of dimerization. The protein kinases can be expressed in cell free translation systems and directly interrogated in competitive binding assays with a split-luciferase reassembly dependent luminescent readout. We demonstrate that an inhibitor screen starting from stored mRNA can be performed in ~3 hours. Thus this approach has the potential to provide a simpler alternative to the existing small-molecule competition based approach previously reported by Fabian et al.^{10, 11} We anticipate that alternate split-reporter systems can be constructed that result in an autoinhibited state where the signal is off, which can be turn-on by addition of a small molecule ligand.⁷⁶ Finally, this CID based approach also has the added benefit that it does not require extensive propagation of cells, phage

display, quantitative PCR, or halo based assays but relies on widely utilized luminescence measurements.

We have demonstrated that the split-protein based three-hybrid approach can be used to interrogate the relative binding affinities for a large number of small molecule inhibitors that target the active or allosteric site for a particular kinase. We have also demonstrated that staurosporine binding serves as a potential surrogate for examining the role of particular residues or domains with regard to the ATP binding cleft of a protein kinase. However, our methodology is not without certain limitations. For one, the actual phosphorylation state of a particular kinase is not known, which is also true for the aforementioned large scale profiling methods, where the protein kinases are expressed in non-native hosts such as bacteria or yeast. Another potential drawback is that the modified staurosporine will likely be unable to target protein kinases that have poor affinity for staurosporine, such as mitogen-activated protein kinases (MAPKs). However, we anticipate that replacing staurosporine with appropriate kinase inhibitors, such as sunitinib or dasatinib, will in the long term allow us to sample a large fraction of the kinome.¹⁵ Future experiments will also focus upon inclusion of full-length kinases, especially those containing known regulatory domains, alongside the catalytic core domain as we have shown for AKT. This will aid in further validating this three-hybrid system and potentially allow for identifying non-active site targeted inhibitors for a particular kinase. Finally, certain small molecules that potently inhibit luciferase activity will not be amenable for the split-luciferase based three-hybrid approach, but we anticipate that for such luciferase inhibitors⁴⁹ a similar strategy can be realized with alternate split-proteins, such as β -lactamase.^{27,36,38} Thus given the advantages and disadvantages, on balance this new cell-free approach may have the potential to provide a simple and rapid competition based method to interrogate inhibitors of protein kinases. Future experiments will explore cell based versions of this split-protein three hybrid system for validating kinase inhibitors in a physiological context. More generally, this three hybrid approach potentially provides a general method to utilize split-proteins for interrogating inhibitors that target small molecule/enzyme interactions as well as providing a new class of chemical inducers of dimerization that utilize coiled-coil handles, which may have utility outside a cell.

Materials and Methods

General Materials

All reagents were from Sigma-Aldrich unless otherwise noted. Enzymes and oligonucleotide primers for cloning were purchased from NEB and IDT respectively. $\text{Mg}(\text{OAc})_2$ was obtained from EM Sciences and Tris.HCl was obtained from Research Products International Corp. All translation materials were from either Promega or Luceome Biotechnologies.

Clone Construction and mRNA Synthesis

All clones were prepared by generating DNA strands encoding appropriate protein fragments by PCR and cloning them into modified pETDuet-1 vector (Novagen) or pRSFDuet-1 vector (Novagen) using standard cloning techniques. Each Cfluc or Nfluc domain was connected to the luciferase fragments by a 13 amino acid $(\text{GGS})_n$ linker. All clones were verified by dideoxyoligonucleotide sequencing. All protein kinase DNA fragments for cloning were generated by PCR using relevant primers and specific DNA template sequences. A list of specific residues used in each construct and NCBI reference sequence numbers can be found in the Supplementary Information, Table S1. To generate mRNAs of each clone, PCR fragments of each cloned fusion construct were produced from a forward primer containing a T7 RNA polymerase promoter and mammalian Kozak

sequence and a reverse primer encoding a 3' hairpin loop.²⁷ These PCR products were subsequently used as templates for *in vitro* mRNA synthesis using a RiboMax Large Scale RNA Production System-T7 (Promega) according to the manufacturer's protocols.

Mutagenesis of Kinase Domains

Kinase activating mutations used in this study were created using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocols. Each complementary forward and reverse mutagenesis primer consisted of 13 codons: the site of mutagenesis flanked by 6 codons on both the 5' and 3' ends. All mutagenesis products were verified by dideoxyoligonucleotide sequencing and the corresponding mRNA was produced as described for the wild-type clones.

Synthesis of Peptide-Inhibitor Conjugate

All small molecules and peptide conjugates used in the present study have been previously described in the literature.^{28,47} Staurosporine was either purchased from LC Labs or produced from *Lentzea albida* (Supplementary Information).

Small Molecule-Induced Ffluc Reassembly and Small Molecule Inhibition

Split-firefly luciferase mRNA constructs were translated in Flexi-Rabbit Reticulocyte Lysate (Promega) according to manufacturer's protocol and assayed for luminescence activity as previously reported.²⁷ 25 μ L translation reactions were performed in duplicate using 0.5 pmol of Fos-Nfluc mRNA, 0.2 pmol of Cfluc-Kinase mRNA, 2 mM DTT, and 0.5 μ L RNasin Plus (Promega). Alternate constructs Cfluc-Fos and PKA-Nfluc were tested under identical conditions using 0.5 pmol and 0.2 pmol mRNA respectively. After translations were incubated at 30 °C for 90 min, 1 μ L of 3.125 μ M **2** in buffer A (6.76 mM TrisHCl, 3.38 mM Mg(OAc)₂, pH 7.45) was added to 24 μ L of each reaction for complex formation. Similarly, 1 μ L of buffer A, Jun, or St β A at 3.125 μ M in buffer A were added to 24 μ L of a reaction as negative controls. In initial studies, samples were equilibrated at 4 °C for 4 hr before being treated with inhibitor. For positive control (with **2**) and negative control (only buffer A) samples, 24 μ L of each reaction was treated by addition of 1 μ L DMSO. Inhibition by PKC-412 (Alexis), Akt1/2 (Sigma), and staurosporine was tested by adding 1 μ L of inhibitor at appropriate concentrations in DMSO to 24 μ L mixtures of lysate containing **2** at 125 nM. All samples were equilibrated for 1 hr in the dark at room temperature upon addition of DMSO or inhibitor. The final concentration of **2** prior to measurement of luminescence was 120 nM. Luciferase activity was determined by mixing 20 μ L lysate with 80 μ L luciferase assay reagent incubating for 1 min, and measuring luminescence using a Turner Biosystems 20/20n luminometer with a 10 sec integration time.

Small Molecule Kinase Inhibitor Profiling against PKA, AKT1, FGFR1/FLT2, and PIM1

For plate-based small molecule screens, translations were scaled to provide necessary reagents for duplicate reactions in 96-well plates. Each Cfluc-Kinase was translated separately along with Fos-Nfluc using the conditions listed above. After translation and incubation at 30 °C for 90 min, several 24 μ L aliquots of lysate were supplemented with 1 μ L of buffer A and set aside as negative controls. The remaining lysate was treated with **2** to a final concentration of 125 nM. 24 μ L aliquots of lysate treated with **2** were added to each well of a 96-well Lumitrac 200 plate (Grenier Bio-one) containing 1 μ L DMSO (for positive controls) or 250 μ M inhibitor (final concentration of 10 μ M). 76 of the inhibitors used on each plate were from the Tocris Kinase Inhibitor Toolbox (Tocris Bioscience). Four inhibitors, PKC-412 (LC Labs), Sunitinib (LC Labs), Flavopiridol (Alexis), and Roscovitine (LC Labs), were also included. A list of all compounds tested, references, and their location on each plate can be found in the Supplementary Information (Table S2). The final

concentrations of **2** and inhibitor were 120 nM and 10 μ M respectively. Plates were covered with foil and equilibrated for 1 hour at room temperature. The Fos-Nfluc with Cfluc-Jun control was prepared in parallel under identical conditions, excluding the addition of **2**. Using a Centro XS LB 960 plate reader (Berthold Technologies), 80 μ L of luciferin assay reagent was injected into each well containing sample and luminescence immediately measured with a 1 sec integration time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the NSF (CHE-0548264) and NIH (R21CA141974 and R01AI068414) for partial support of this research. The authors would like to thank members of the Ghosh lab and Dr. Reena Zutshi for helpful discussions. Molecular structures were rendered using PyMol; DeLano, W. L. (www.pymol.org).

References

1. Knight ZA, Lin H, Shokat KM. Nat Rev Cancer. 2010; 10:130–137. [PubMed: 20094047]
2. Zhang JM, Yang PL, Gray NS. Nat Rev Cancer. 2009; 9:28–39. [PubMed: 19104514]
3. Lee J, Laydon J, McDonnell P, Gallagher T, Kumar S, Green D, McNulty D, Blumenthal M, Heys J, Landvatter S. Nature. 1994; 372:739–746. [PubMed: 7997261]
4. Kyriakis JM, Avruch J. J Biol Chem. 1996; 271:24313–24316. [PubMed: 8798679]
5. Hotamisligil G. Nature. 2006; 444:860–867. [PubMed: 17167474]
6. Cohen P. Nat Rev Drug Discov. 2002; 1:309–315. [PubMed: 12120282]
7. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. New Engl J Med. 2001; 344:1031–1037. [PubMed: 11287972]
8. Noble M, Endicott J, Johnson L. Science. 2004; 303:1800–1805. [PubMed: 15031492]
9. Manning G, Whyte D, Martinez R, Hunter T, Sudarsanam S. Science. 2002; 298:1912–1934. [PubMed: 12471243]
10. Fabian MA, et al. Nat Biotechnol. 2005; 23:329–336. [PubMed: 15711537]
11. Karaman M, et al. Nat Biotechnol. 2008; 26:127–132. [PubMed: 18183025]
12. Bain J, Plater L, Elliott M, Shpiro N, Hastie C, McLauchlan H, Klevernic I, Arthur J, Alessi D, Cohen P. Biochem J. 2007; 408:297–315. [PubMed: 17850214]
13. Bishop AC, Ubersax JA, Petsch DT, Matheos DP, Gray NS, Blethrow J, Shimizu E, Tsien JZ, Schultz PG, Rose MD, Wood JL, Morgan DO, Shokat KM. Nature. 2000; 407:395–401. [PubMed: 11014197]
14. Knight ZA, Shokat KM. Chem Biol. 2005; 12:621–637. [PubMed: 15975507]
15. Krishnamurthy R, Maly DJ. Comb Chem High Throughput Screen. 2007; 10:652–666. [PubMed: 18045078]
16. Hastie CJ, McLauchlan HJ, Cohen P. Nat Protoc. 2006; 1:968–971. [PubMed: 17406331]
17. Bain J, McLauchlan H, Elliott M, Cohen P. Biochem J. 2003; 371:199–204. [PubMed: 12534346]
18. Davies S, Reddy H, Caivano M, Cohen P. Biochem J. 2000; 351:95–105. [PubMed: 10998351]
19. Corson TW, Aberle N, Crews CM. ACS Chem Biol. 2008; 3:677–692. [PubMed: 19112665]
20. Clark DD, Peterson BR. ChemBioChem. 2003; 4:101–107. [PubMed: 12512083]
21. Becker F, et al. Chem Biol. 2004; 11:211–223. [PubMed: 15123283]
22. Spencer DM, Wandless TJ, Schreiber SL, Crabtree GR. Science. 1993; 262:1019–1024. [PubMed: 7694365]
23. Belshaw PJ, Ho SN, Crabtree GR, Schreiber SL. Proc Natl Acad Sci USA. 1996; 93:4604–4607. [PubMed: 8643450]

24. Licitra EJ, Liu JO. *Proc Natl Acad Sci USA*. 1996; 93:12817–12821. [PubMed: 8917502]
25. Lin HN, Abida WM, Sauer RT, Cornish VW. *J Am Chem Soc*. 2000; 122:4247–4248.
26. Hussey SL, Muddana SS, Peterson BR. *J Am Chem Soc*. 2003; 125:3692–3693. [PubMed: 12656587]
27. Porter J, Stains C, Jester B, Ghosh I. *J Am Chem Soc*. 2008; 130:6488–6497. [PubMed: 18444624]
28. Meyer S, Shomin C, Gaj T, Ghosh I. *J Am Chem Soc*. 2007; 129:13812–13813. [PubMed: 17944472]
29. Michnick SW, Ear PH, Manderson EN, Remy I, Stefan E. *Nat Rev Drug Discov*. 2007; 6:569–582. [PubMed: 17599086]
30. Johnsson N, Varshavsky A. *Proc Natl Acad Sci USA*. 1994; 91:10340–10344. [PubMed: 7937952]
31. Dirnberger D, Unsin G, Schlenker S, Reichel C. *ChemBioChem*. 2006; 7:936–942. [PubMed: 16680785]
32. Ghosh I, Hamilton AD, Regan L. *J Am Chem Soc*. 2000; 122:5658–5659.
33. Hu CD, Kerppola TK. *Nat Biotechnol*. 2003; 21:539–545. [PubMed: 12692560]
34. Stains CI, Porter JR, Ooi AT, Segal DJ, Ghosh I. *J Am Chem Soc*. 2005; 127:10782–10783. [PubMed: 16076155]
35. Pelletier JN, Campbell-Valois FX, Michnick SW. *Proc Natl Acad Sci USA*. 1998; 95:12141–12146. [PubMed: 9770453]
36. Galarneau A, Primeau M, Trudeau LE, Michnick SW. *Nat Biotechnol*. 2002; 20:619–622. [PubMed: 12042868]
37. Ooi AT, Stains CI, Ghosh I, Segal DJ. *Biochemistry*. 2006; 45:3620–3625. [PubMed: 16533044]
38. Porter JR, Stains CI, Segal DJ, Ghosh I. *Anal Chem*. 2007; 79:6702–6708. [PubMed: 17685552]
39. Luker KE, Smith MCP, Luker GD, Gammon ST, Piwnica-Worms H, Piwnica-Worms DP. *Proc Natl Acad Sci USA*. 2004; 101:12288–12293. [PubMed: 15284440]
40. Remy I, Michnick SW. *Nat Methods*. 2006; 3:977–979. [PubMed: 17099704]
41. Lupas A. *Trends Biochem Sci*. 1996; 21:375–382. [PubMed: 8918191]
42. Bromley EHC, Channon K, Moutevelis E, Woolfson DN. *ACS Chem Biol*. 2008; 3:38–50. [PubMed: 18205291]
43. Mason JM, Arndt KM. *ChemBioChem*. 2004; 5:170–176. [PubMed: 14760737]
44. Grigoryan G, Keating AE. *Curr Opin Struct Biol*. 2008; 18:477–483. [PubMed: 18555680]
45. Kodadek T. *Nat Chem Biol*. 2010; 6:162–165. [PubMed: 20154660]
46. Feng BY, Shelat A, Doman TN, Guy RK, Shoichet BK. *Nat Chem Biol*. 2005; 1:146–148. [PubMed: 16408018]
47. Shomin CD, Meyer SC, Ghosh I. *Bioorg Med Chem*. 2009; 17:6196–6202. [PubMed: 19674907]
48. Magliery TJ, Wilson CGM, Pan WL, Mishler D, Ghosh I, Hamilton AD, Regan L. *J Am Chem Soc*. 2005; 127:146–157. [PubMed: 15631464]
49. Auld DS, Southall NT, Jadhav A, Johnson RL, Diller DJ, Simeonov A, Austin CP, Inglese J. *J Med Chem*. 2008; 51:2372–2386. [PubMed: 18363348]
50. Jacobs M, Black J, Futer O, Swenson L, Hare B, Fleming M, Saxena K. *J Biol Chem*. 2005; 280:13728–13734. [PubMed: 15657054]
51. Yang L, Dan HC, Sun M, Liu QY, Sun XM, Feldman RI, Hamilton AD, Polokoff M, Nicosia SV, Herlyn M, Sebt SM, Cheng JQ. *Cancer Res*. 2004; 64:4394–4399. [PubMed: 15231645]
52. Kim D, Sun M, He LL, Zhou QH, Chen J, Sun XM, Bepler G, Sebt SM, Cheng JQ. *J Biol Chem*. 2010; 285:8383–8394. [PubMed: 20068047]
53. Amodeo GA, Rudolph MJ, Tong L. *Nature*. 2007; 449:492–495. [PubMed: 17851534]
54. Xiao B, Heath R, Saiu P, Leiper FC, Leone P, Jing C, Walker PA, Haire L, Eccleston JF, Davis CT, Martin SR, Carling D, Gamblin SJ. *Nature*. 2007; 449:496–500. [PubMed: 17851531]
55. Pelham HRB, Jackson RJ. *Eur J Biochem*. 1976; 67:247–256. [PubMed: 823012]
56. Hubbard SR, Wei L, Elis L, Hendrickson WA. *Nature*. 1994; 372:746–754. [PubMed: 7997262]
57. Huse M, Kuriyan J. *Cell*. 2002; 109:275–282. [PubMed: 12015977]
58. Lochhead PA. *Sci Signal*. 2009; 2:pe4. [PubMed: 19155529]

59. Seger R, Krebs EG. *FASEB J.* 1995; 9:726–735. [PubMed: 7601337]
60. Russo AA, Tong L, Lee JO, Jeffrey PD, Pavletich NP. *Nature.* 1998; 395:237–243. [PubMed: 9751050]
61. Johnson LN, Noble MEM, Owen DJ. *Cell.* 1996; 85:149–158. [PubMed: 8612268]
62. Nolen B, Taylor S, Ghosh G. *Mol Cell.* 2004; 15:661–675. [PubMed: 15350212]
63. Levin DH, Ranu RS, Ernst V, Fifer MA, London IM. *Proc Natl Acad Sci USA.* 1975; 72:4849–4853. [PubMed: 174078]
64. Shibuya EK, Ruderman JV. *Mol Biol Cell.* 1993; 4:781–790. [PubMed: 8241566]
65. Manser E, Huang HY, Loo TH, Chen XQ, Dong JM, Leung T, Lim L. *Mol Cell Biol.* 1997; 17:1129–1143. [PubMed: 9032240]
66. Sanders LC, Matsumura F, Bokoch GM, de Lanerolle P. *Science.* 1999; 283:2083–2085. [PubMed: 10092231]
67. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. *EMBO J.* 1996; 15:6541–6551. [PubMed: 8978681]
68. Yang J, Cron P, Thompson V, Good VM, Hess D, Hemmings BA, Barford D. *Mol Cell.* 2002; 9:1227–1240. [PubMed: 12086620]
69. Frodin M, Jensen CJ, Merienne K, Gammeltoft S. *EMBO J.* 2000; 19:2924–2934. [PubMed: 10856237]
70. Biondi RM, Kieloch A, Currie RA, Deak M, Alessi DR. *EMBO J.* 2001; 20:4380–4390. [PubMed: 11500365]
71. Krishnamurthy R, Maly DJ. *ACS Chem Biol.* 2010; 5:121–138. [PubMed: 20044834]
72. Lindsley CW, Zhao ZJ, Leister WH, Robinson RG, Barnett SF, Defeo-Jones D, Jones RE, Hartman GD, Huff JR, Huber HE, Duggan ME. *Bioorg Med Chem Lett.* 2005; 15:761–764. [PubMed: 15664853]
73. Zhao ZJ, William HL, Robinson RG, Barnett SF, Defeo-Jones D, Jones RE, Hartman GD, Huff JR, Huber HE, Duggan ME, Lindsley CW. *Bioorg Med Chem Lett.* 2005; 15:905–909. [PubMed: 15686884]
74. Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, Kahana JA, Kral AM, Leander K, Lee LL, Malinowski J, McAvoy EM, Nahas DD, Robinson RG, Huber HE. *Biochem J.* 2005; 385:399–408. [PubMed: 15456405]
75. Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJC, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA. *J Biol Chem.* 1997; 272:31515–31524. [PubMed: 9395488]
76. Shekhawat SS, Porter JR, Sriprasad A, Ghosh I. *J Am Chem Soc.* 2009; 131:15284–15290. [PubMed: 19803505]

Figure 1.

Design of a coiled-coil-enabled three-hybrid split-protein system for kinases. The two fragments of split-luciferase are attached to a coiled-coil Fos and a kinase (Fos-Nfluc and Cfluc-Jun). Ternary complex formation results in the reassembly of active luciferase from the two fragments mediated by the chemical inducer of dimerization (CID) comprising staurosporine conjugated to a coiled-coil peptide, Jun (Jun-St) in yellow. The addition of a CID competitive small molecule (gray) reverses luciferase reassembly.

Figure 3.

Generality of the three-hybrid system. (A) A representation of the human kinome with the seven major groups. (B) Structure of the staurosporine derivative PKC-412 (4). (C) Ten representative protein kinases from six groups. Translations of the two split-luciferase halves appended to the indicated protein kinases was equilibrated with 2 and treated with either no inhibitor (blue) or 10 μ M 4 (pink) to determine if it could form an active ternary complex with the Jun-staurosporine CID and be competitively displaced by 4.

Figure 4.

A small molecule profile of 80-inhibitors against (A) PKA, (B) AKT1, (C) FGFR1/FLT2, and (D) PIM1. Each X, Y coordinate represents an individual small molecule present at 10 μM with the degree of inhibition plotted on the Z-axis. (E) Structures of select molecules used in the screen.

Figure 5.

Testing the effect of activating and inactivating protein kinase mutations on inhibitor binding. Model of protein kinase and mutational analysis of regulatory residues for (A) PAK1 (PDB 1YHV) and (B) AKT2 (PDB 1O6K). Both models are shown with the sites of phosphorylation highlighted (red) and an ATP-analog (yellow) bound in the active site. The relative luminescence of the three-hybrid complex upon the addition of the CID indicates aspartic acid (D) and glutamic acid (E) mutants exhibit an increase in relative luminescence relative to the wild-type and alanine (A) mutants.

Figure 6.

Testing allosteric inhibition of a protein kinase in the three-hybrid system. (A) A proposed model of allosteric inhibitors of AKT1, where an inhibitor may act by binding outside the active site at a pleckstrin homology (PH) domain to induce an inactive conformation. (B) Structure of the allosteric AKT1 inhibitor Akt1/2 (13). (C) Domain map of two different protein kinase constructs containing only the kinase domain and c-terminal region (AKT1) or the full-length protein including the PH domain (flAKT1). (E) Inhibition of AKT1 and flAKT1 by either an ATP-competitive inhibitor 4 or the reported allosteric inhibitors 13 and 12.