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Ligand-Gated Split-Kinases

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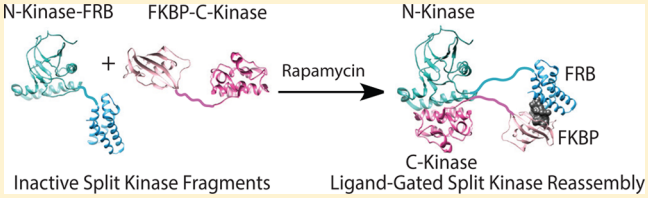
Supporting Information

ABSTRACT: The activity of protein kinases are naturally gated by a variety of physiochemical inputs, such as phosphorylation, metal ions, and small molecules. In order to design protein kinases that can be gated by user-defined inputs, we describe a sequence dissimilarity based approach for identifying sites in protein kinases that accommodate 25-residue loop insertion while retaining catalytic activity. We further demonstrate that the successful loop insertion mutants provide guidance for the dissection of protein kinases into two fragments that cannot spontaneously assemble and are thus inactive but can be converted into ligand-gated catalytically active split-protein kinases. We successfully demonstrate the feasibility of this approach with Lyn, Fak, Src, and PKA, which suggests potential generality.



INTRODUCTION

The tandem activity of protein kinases and phosphatases catalyze the reversible phosphorylation of a host of client proteins and thereby exert control over their temporal activity and spatial localization. The greater than 500 human protein kinases and their isoforms control a vast array of signaling pathways and cellular events from cell division to cell death.[1] The aberrant function of many protein kinases has been associated with a multitude of diseases, such as cancer,[2,3] metabolic disorders,[4] and inflammation;[5] hence, protein kinases have emerged as important therapeutic targets. However, deciphering the specific role of a kinase of interest remains challenging. Though powerful, siRNA-based genetic knockdown methods, which provide insight regarding the function of a specific protein kinase, presently lack spatial and temporal control and often fall prey to compensatory cellular mechanisms. Toward the goal of establishing immediate temporal control of a specific protein kinase, three elegant methods have been developed that seek to either turn a specific kinase on or off.

Shokat and co-workers pioneered a pharmacological knockdown approach for the selective control over designed kinases by mutation of the gatekeeper residue adjacent to the kinase ATP-binding cleft.[6,7] These mutations expanded the ATP-binding site while native kinase activity is maintained; hence, inhibitors could be designed with complementary bulky substituents by selectively targeting and inhibiting the designed mutant. This chemical genetic approach, termed inhibitor-sensitive kinase alleles, has been widely applied in vivo for turning off a specific protein kinase.[8] A more recent protein engineering approach has been described by Hahn and co-workers that provides allosteric control over kinase activity.[9,10] In this approach, a modified FK506 binding protein (FKBP) was successfully inserted into a highly conserved region of the catalytic domain of a protein kinase, which coupled native β-strands of the protein kinase with that of FKBP. The FKBP insertion rendered the kinase inactive, likely due to disruption of the tertiary structure. However, upon the addition of the small molecule rapamycin and FKBP12−rapamycin binding protein (FRB), the ternary complex likely restored catalytic activity through the reestablishment of the native tertiary structure of the disrupted catalytic domain. This approach has recently been further modified, where redesigned FKBP and FRB are inserted in tandem into the kinase catalytic domain and respond to rapamycin.[11] A third approach for specific control over a protein kinase, pioneered by Chin and co-workers, incorporated a genetically encoded non-natural photocaged lysine implicated in catalysis that renders the kinase inactive.[12] Irradiation of cells expressing the mutant kinase relieved the photocaging of lysine, leading to gain of kinase activity and downstream signaling. Each of these innovative approaches, focusing on rendering a specific kinase inactive or active, has merits for interrogating a particular biological pathway. Several complementary approaches targeted to a specific kinase will certainly help in resolving any method-dependent ambiguities. Furthermore, new approaches that provide the potential for ligand-induced gating of the activity of not only one but multiple kinases will allow for understanding and engineering of more complex signal transduction pathways.[13,14]

In order to impart temporal and spatial control over multiple kinases, we became interested in exploring the possibility of constructing ligand-controlled split-protein kinases (split-kinases). Each fragment of a split-kinase would be inactive until they reassembled with attendant restoration of catalytic activity. We envisioned that an appropriately fragmented kinase could be conditionally reassembled or gated by a stimulus, such

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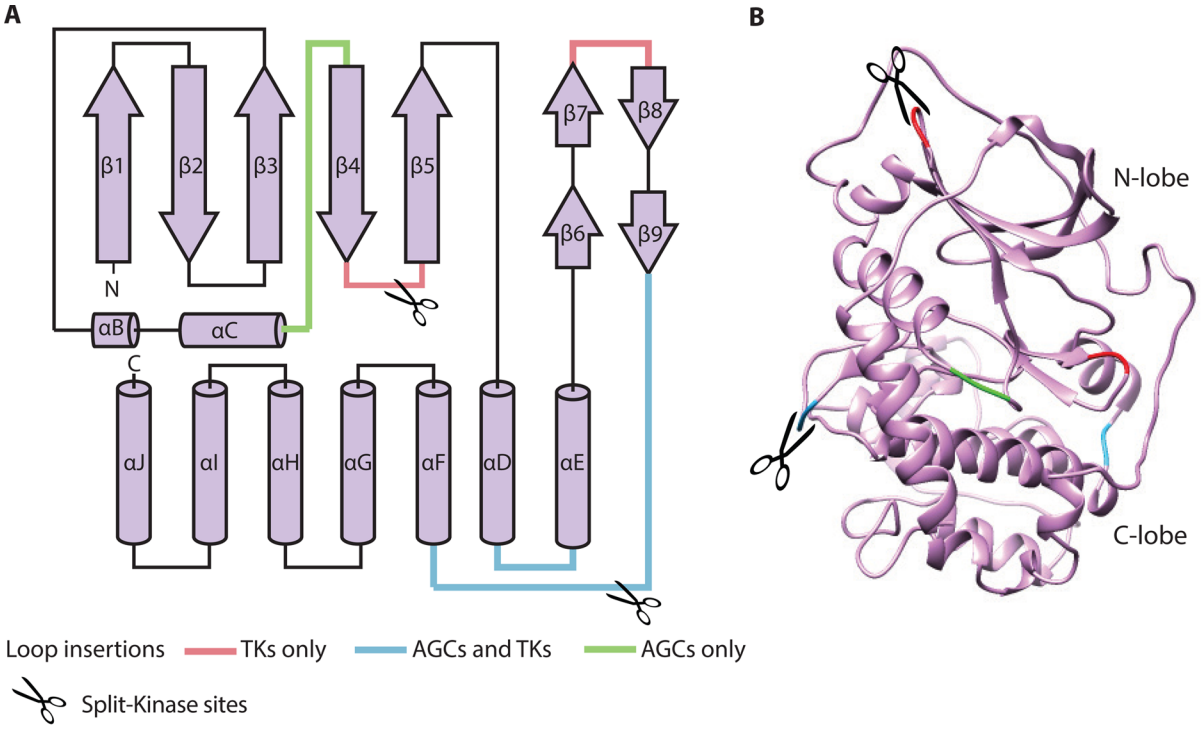

C

TK β4-β5 Alignment

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Lyn     HDKLVRLYAVVTR--EEP------IYIITEYMAKGSL
FAK     HPHIVKLIGVITE---NP------VWIIMELCTLGEL
Src     HEKLVQLYAVVS---EEP------IYIVTEYMSKGSL
JAK1b   HENIVKYKGICTEDGGNG------IKLIMEFLPSGSL
Tyk2b   HEHIIKYKGCCEDQGEKS------LQLVMEYVPLGSL
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TK β9-αF Alignment

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Lyn     KIADFGLARVIE---DNEYT
FAK     KLGDFGLSRYME---DSTYY
Src     KVADFGLARLIE---DNEYT
FRK     KVADFGLARVFKVDNEDIYE
EphB4   KVSDFGLSRFLEENSSDPTY
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AGC β9-αF Alignment

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PKA       PENLLIDQQGYIQVTDFGFAKRVK----------------------------------------------------GRTWTLCGTPEYLAPEII
MAST1     PDNLLITSMGHIKLTDFGLSKMGLMS-LTTNLYE-------------------------------GHIEKDAREFLDKQVCGTPEYIAPEVI
LATS1     PDNILIDRDGHIKLTDFGLCTGFRWT-HDSKYYQSGDHPRQDSMDFSNEWGDPSSCRCGDRLKPLERRAARQHQRCLAHSLVGTPNYIAPEVL
NDR1      PDNLLLDSKGHVKLSDFGLCTGLKKA-HRTEFYRNLNHSLPSDFTFQNMNSK------------RKAETWKRNRRQLAFSTVGTPDYIAPEVF
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Figure 1. Identification of loop insertion and fragmentation sites. (A) Topological representation of the catalytic domain of a generic protein kinase. Sites chosen for loop insertion are depicted in red for the tyrosine kinase (TK) group, green for the AGC group, and blue for sites common to both TK and AGC kinases. Sites ultimately identfied for fragmentation are represented with scissors. (B) Three dimensional representation of a protein kinase (PDB ID 1ATP) with loop insertion sites depicted using the same colors as in panel A. (C) Partial alignment of TK and AGC kinases shows insertion sites (in red) chosen on the basis of gaps in the alignment.

as the addition of a small molecule ligand or light.[15,16] Gated split-proteins that can conditionally reassemble include split-ubiquitin,[17] split-GFP,[18] split-lactamase,[19] and split-luciferase,[20−22] among many others. Most split-proteins to date have been primarily developed for the interrogation and detection of macromolecular interactions. In another front, Wells and co-workers have utilized a rapamycin-gated split-TEV protease for precisely timing caspase cleavage events to interrogate apoptosis.[23] Split-proteins are clearly useful;[24] however, the identification of appropriate dissection sites and methods for identification of functionally reassembled proteins remains challenging. For example, the dissection of firefly luciferase utilized incremental truncation,[20] wherein the optimal protein fragments would perhaps have been difficult to anticipate by rational design even with a readily measurable enzymatic output.

Toward the long-term goal of identifying potentially general sites for constructing ligand-gated split-kinases, we hypothesized that the identification of sequences in the catalytic domain of protein kinases that harbor significant dissimilarities would guide the design of catalytic domains that retain activity with deliberate loop insertions of 25 residues. We further hypothesized that the successful loop insertions would in turn identify sites tolerant to fragmentation to ultimately afford ligand-gated split-kinases. Herein, we test our hypotheses initially with Lyn, a tyrosine kinase (TK). Subsequently, we demonstrate the generality of our approach by showing that it can be extended to other members of the TK and AGC groups. To our knowledge, these are the first examples of the rational design of ligand-activated split-protein kinases.

RESULTS AND DISCUSSION

Choice of Loop Insertions and Fragmentation Sites.

Human protein kinases are divided into several groups, such as TKs and AGCs based on sequence similarity.[1] The catalytic domain of kinases (Figure 1A,B, labeled following PKA

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convention) have an N-terminal lobe composed primarily of β-sheets and a C-terminal lobe that is primarily composed of α-helices. The catalytic cleft that binds ATP and the substrate (Figure 1B) is located at the juncture between the two lobes. We aligned the catalytic domain of human protein TK and AGC kinases using ClustalO.[25,26] Loop insertion sites for TK and AGC group kinases were chosen on the basis of dissimilarity, particularly insertions, observed in kinase sequences (Supporting Information, Figures S1 and S2). Representative sequences from within the TKs show that Jak1b and Tyk2b can accommodate a potential insertion when compared to Lyn, FAK, and Src at the $\beta4-\beta5$ junction (Figure 1C). Similarly, the $\beta9-\alpha$F loop accommodates insertions when comparing FRK and EphB4 to Lyn, FAK, and Src (Figure 1A,C). When considering the AGCs, LATS1 and NDR1 can potentially accommodate insertions when compared to PKA and MAST1. On the basis of this idea of known areas with dissimilarity within kinase groups, initial loop insertion and thereby potential fragmentation sites were identified between $\beta4-\beta5$, αD$-\alpha$E, $\beta7-\beta8$, and $\beta9-\alpha$F (activation loop) for the TKs, Lyn and FAK (Figure 1A). Similarly, 25 residue insertions were engineered between αC$-\beta4$, αD$-\alpha$E, and $\beta9-\alpha$F for the AGC group kinase, PKA (Figure 1A). The final loop insertion sites for testing activity were chosen such that the respective domains N- and C-terminal to the loop contained >75 residues, lengths often observed for small independently folded proteins or protein domains.[27,28]

Loop Insertion Mutants of Lyn. We began testing our hypothesis toward the generation of split-kinases with Lyn, a member of the TK group. Lyn is vital for B-cell proliferation, differentiation, and apoptosis.[29] The first hurdle that needed to be overcome was the ability to produce and purify functional Lyn. In previous work we have developed numerous split-protein assays utilizing cell free systems, such as rabbit reticulocyte lysate (RRL) and wheat germ lysate (WGL).[30] Anticipating the need for rapid expression, purification, and testing for catalytic activity of engineered kinases we incorporated a C-terminal hexa-histidine tag in Lyn. The catalytic domain of Lyn (residues 211−512), hereafter referred to as Lyn-WT, was expressed in cell-free systems using RRL, and methods were optimized for purification of expressed protein using Ni-NTA affinity chromatography and subsequent interrogation of activity using ^{32}P incorporation assays. Attempts to elute proteins, synthesized at 1 pmol mRNA scale, from resin using imidazole or further purification rendered the kinase inactive (Supporting Information, Figure S3). Thus, all subsequent ^{32}P incorporation assays were carried out on the resin. Lyn-WT and all the insertion mutants were also engineered to incorporate an N-terminal fragment of firefly luciferase, Cfluc, as this would allow for testing of direct small molecule binding using a previously developed three-hybrid split-luciferase kinase assay.[31]

Based on our sequence analysis (Figure 1), the 25-residue loop insertions (CGRGGSGGGGSGGGGSVDGGSGGQL), were incorporated between R311−E312, D334−E335, S378−L379, and E393−D394 (Supporting Information, Figure S1). Subsequently, all the resulting Lyn constructs were expressed in RRL and the resin-bound activity of the wild type and insertion mutants of Lyn were assayed by phosphorylation of the Lyn substrate (EDPIYEFLPAKKK). Results from ^{32}P incorporation assays (Figure 2A) clearly demonstrate that the loop insertions between R311−E312 and S378−L379 are 10-fold less active than the Lyn-WT. Interestingly, the loop insertion in the activation loop (E393−D394) showed activities very similar to that of Lyn-WT. It is possible that the activation loop of most kinases, often unresolved in many crystal structures, is dynamic and perhaps structurally predisposed for accommodating insertions.

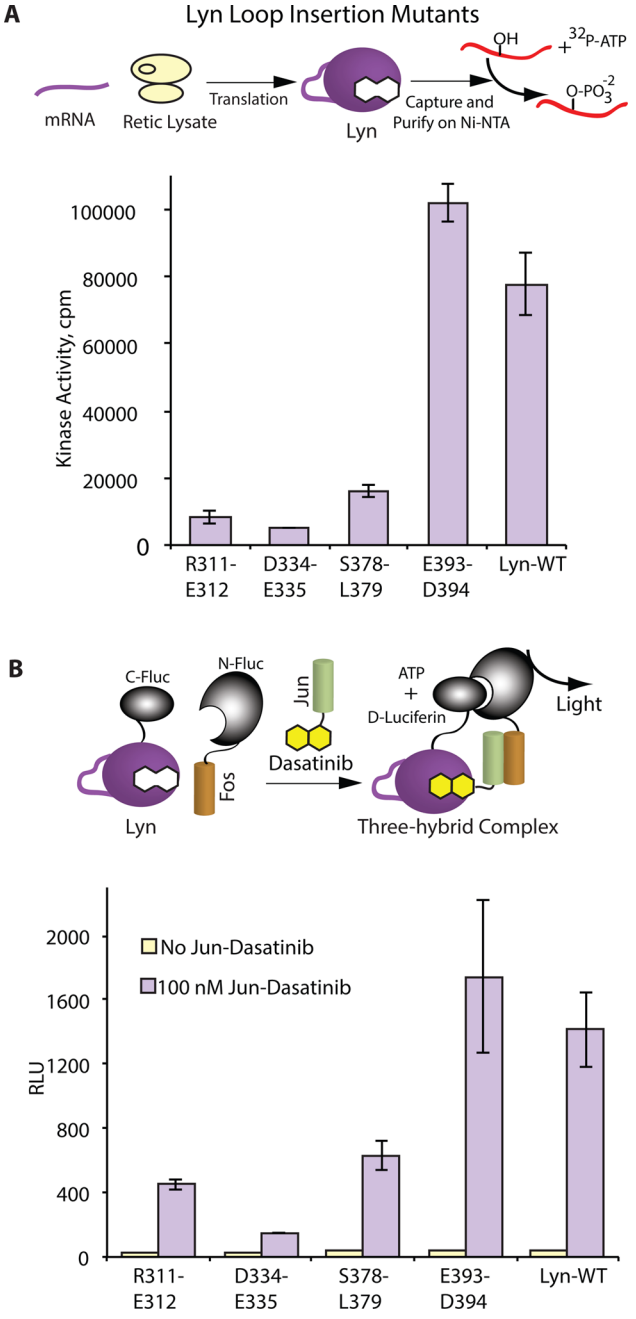

Figure 2. Loop insertions in Lyn. (A) Results from kinase assays for a 25-residue loop insertion at the positions in the catalytic domain of Lyn as indicated on the *x*-axis. The loop insertion mutants were expressed in vitro, purified, and ^{32}P incorporation was measured with its substrate (EDPIYEFLPAKKK) and ^{32}P-ATP. Activity data are not normalized and are presented after background correction of equivalent treatment of rabbit reticulocyte lysate. (B) Luminescence results for Jun−dasatinib binding to loop insertion mutants and Lyn-WT in a three-hybrid split-luciferase system.

Split-Luciferase Assay of Lyn Loop Insertion Mutants. Having established that several of the Lyn insertion mutants retained significant kinase activity, we next set out to

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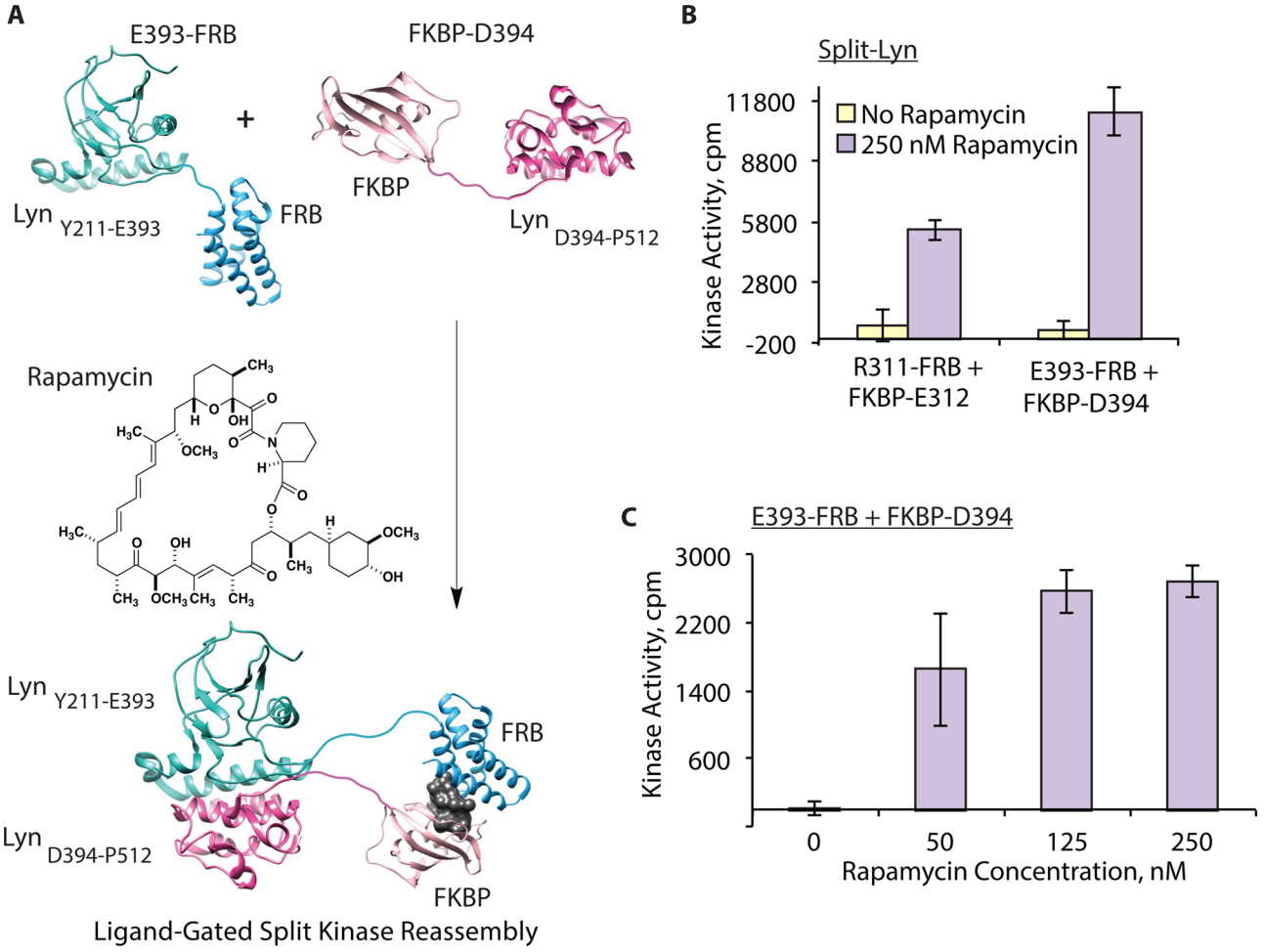

Figure 3. Split-Lyn kinase. (A) The FRB domain (blue) was attached to the N-terminal fragment of Lyn (green) through a 25-residue linker. The C-terminal domain (pink) of Lyn was attached to FKBP (light pink). The chemical inducer of dimerization, rapamycin, results in the formation of the ternary complex FKBP−Rap−FRB, leading to the reassembly of fragmented Lyn. (B) Activity assays for two different split-Lyn kinases at the indicated fragmentation sites clearly demonstrate that kinase activity is recovered only in the presence of added rapamycin. (C) Activity assay for the split-Lyn (E393−D394) at varying rapamycin concentrations. Data from activity assays are not normalized and are presented after background correction of equivalent treatment of rabbit reticulocyte lysate.

interrogate whether loop insertions would impact the ability of the mutant kinases to bind a known small molecule inhibitor, dasatinib. Dasatinib is a promiscuous type I inhibitor of TK group that necessarily requires a structured ATP binding pocket. We tested dasatinib binding to Lyn variants in a three-hybrid split-luciferase assay (Figure 2B).[31,32] The C-terminal fragment of firefly luciferase attached to the N-termini of Lyn-WT was coexpressed with Fos-Nfluc in RRL. Jun-conjugated dasatinib was added to promote luciferase reassembly. The luminescence from the three-hybrid assay (Figure 2B) of Lyn-WT and the loop insertion mutants R311−E312, S378−L379, and E393−D394 were found to be in excellent agreement with the kinase activity assays, suggesting that kinase activity and type I inhibitor binding potentially report upon the integrity of the tertiary structure of the protein kinase.

Split-Lyn. Having successfully identified loop insertion mutants that retained activity and the ability to bind type I inhibitors, we turned to designing two split-Lyn kinases, targeting the R311−E312 and E393−D394 sites for dissection. For our small molecule dependent gating of the potential split-kinases, we chose the classical rapamycin-induced dimerization of FKBP and FRB.[33−36] FRB was attached to the C-termini of R311 and E393, while FKBP was attached to the N-termini of E312 and D394 fragments, respectively, to yield the potential split-kinases R311−FRB/FKBP−E312 and E393−FRB/FKBP−D394 (Figure 3A). In these experiments, we retained the 25 amino acid loop as the linker that was used for the loop insertion mutants between the fragments and FKBP or FRB. The protein pairs, R311−FRB/FKBP−E312 and E393−FRB/FKBP−D394, were cotranslated in RRL in the presence or absence of 250 nM rapamycin and subsequently purified by Ni-affinity. The results (Figure 3B) clearly showed that in the absence of rapamycin, both of the split-Lyn kinases were catalytically inactive. Catalytic activity was only restored when the split-proteins were translated in the presence of rapamycin and that the observed kinase activity was dependent upon rapamycin concentration (Figure 3C). The split-Lyn (E393−D394), with the dissection site in the activation loop, was 3-fold more active than split-Lyn (R311−E312), which reflected our observations from the initial loop insertion studies. When we directly compared the activity of E393−FRB/FKBP−D394 fragments to that of the corresponding E393−D394 loop insertion mutant, we found the split kinase to be 9-fold less active under the purification and assay conditions used initially (Supporting Information, Figure S4). In order to further rule out experimental artifacts, we carried out several independent

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replicates of the rapamycin-dependent activation on separate days using different batches of lysate, mRNA, and rapamycin (Supporting Information, Figures S5 and S6) and found consistent and significant activation of split-Lyn activity in the presence of rapamycin. To our knowledge, this represents the first ligand inducible split protein kinase that has been rationally designed using a sequence dissimilarity based approach that relies on natural sequence variations among related protein kinases.

Split-Fak. Having successfully identified two separate sites for creating split-Lyn kinases, we further hypothesized that the rationale for choice of dissection sites should be applicable to other protein kinases, particularly other TKs. Thus, as a first test for demonstrating generality we chose focal adhesion kinase (Fak), also called Fak1 or protein tyrosine kinase 2 (PTK2), a TK from a different subfamily than Lyn that shares 57% similarity within the catalytic domain. Fak presents a good test case for testing loop insertion mutants, as it has been previously targeted by Hahn and co-workers at a significantly different site (between 442 and 448) using an alternate allosteric mechanism by insertion of FKBP. We did not pursue this specific site for insertion, as it lies within 50 residues of the N-terminus of the catalytic domain.

For Fak, we first introduced a 25-amino acid linker as we had for Lyn, between positions E492−N493, K515−Y516, N557−D558, and E572−D573. We translated the loop insertion mutants and wild-type proteins in RRL and tested kinase activity using a standard TK substrate [poly(Glu:Tyr) polymer with a 4:1 ratio]. The results showed a significant loss in activity for insertion mutants at positions K515−Y516 and N557−D558 compared to wild-type. Mutant E492−N493 showed 3-fold lower activity than the Fak-WT (374−708), while the mutant with the insertion loop in the activation loop (E572−D573) retained activity that was similar to that of Fak-WT (Figure 4A). These results, with regard to the viability of different sites for insertions, are very similar to what we had observed with Lyn and suggests that the two functional sites for loop insertion may perhaps be extendable across the TK group. Having identified successful insertion mutants, we next constructed FKBP- and FRB-containing split-kinases of the two loop insertion mutants (E492−N493 and E572−D573), to yield E492−FRB/FKBP−N493 and E572−FRB/FKBP−D573. We cotranslated each pair of split-Fak kinases in either the presence or absence of 250 nM rapamycin. We found that the two split-Fak kinases, E492−N493 and E572−D573 were 3- and 16-fold more active in the presence of rapamycin [Figures 4B and S7 (Supporting Information)].

Split-Src. Having identified possible general sites for generating ligand-gated TKs between the $\beta4-\beta5$ and $\beta9-\alpha F$ loops for both Lyn and Fak, we decided to directly test our approach by directly targeting these sites on Src and eliminating the loop insertion step. Src displays 84% similarity with Lyn within the catalytic domain, and thus in this example, we decided to directly generate split-Src at positions analogous to Lyn, E334−E335 and E415−D416, respectively. FRB was conjugated to the C-termini of E334 and E415, while FKBP was attached to the N-termini of E335 and D416, respectively, to afford E334−FRB/FKBP−E335 and E415−FRB/FKBP−D416. Proteins were cotranslated in RRL in the presence or absence of 250 nM rapamycin, and activity was measured using the Src substrate, EDPIYEFLPAKKK. The results showed that E334−FRB/FKBP−E335 and E415−FRB/FKBP−D416 are, respectively, 10- and 53-fold more active upon rapamycin addition (Figure 4c). Thus, these results from Lyn, Fak, and Src demonstrate that the $\beta4-\beta5$ and $\beta9-\alpha F$ sites are potentially general sites for fragmentation of the TK group members.

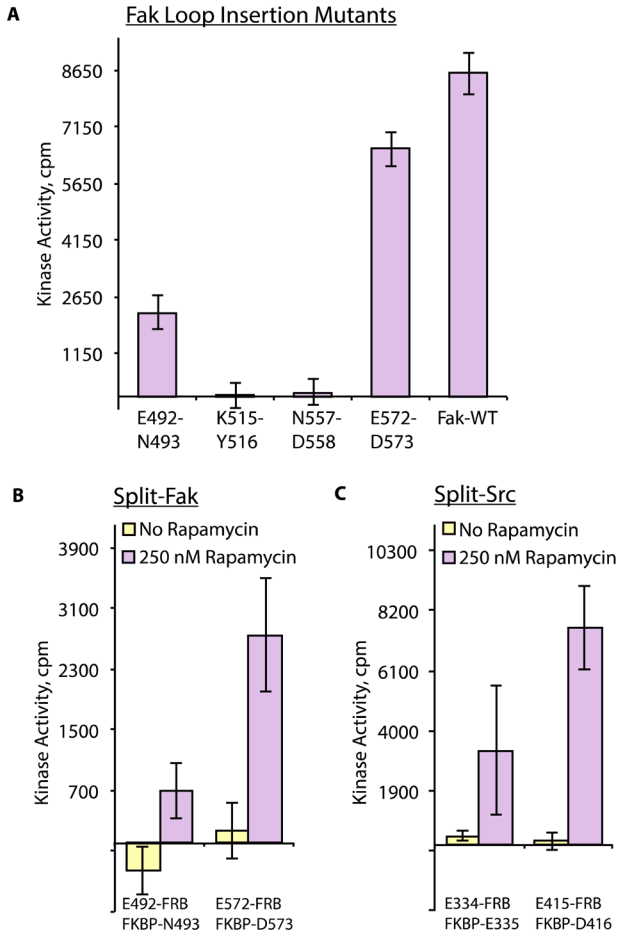

Figure 4. Split-Fak and split-Src. (A) Kinase activity assay results for 25-residue loop insertions in Fak measured using the poly(Glu:Tyr) 4:1 substrate and ^{32}P-ATP. (B) Split-Fak activation with rapamycin measured for two different split-Fak kinases by phosphorylation assay. (C) Split-Src activation with rapamycin measured for two split-Src kinases by phosphorylation assay. Data from activity assays are not normalized and are presented after background correction of equivalent treatment of reticulocyte lysate.

Extension to the AGC Group: Split-PKA. Having provided proof of principle for generating rapamycin inducible split-TKs, we focused upon interrogating whether our methodology could also be applied to the AGC group of kinases and chose the cAMP-dependent protein kinase A, PKA, as a test case. On the basis of our alignments (Supporting Information, Figure S2) we introduced loop insertions in positions N99−F100, R137−F138, and K192−G193 and expressed the proteins in WGL, which provided higher activity than RRL in our hands. We found that only the loop mutant K192−G193 was catalytically active using the GRTGRRNSI substrate and, interestingly, showed similar activity to that of the wild-type (Figure 5A). Since this was the first member of the AGC group tested, we further confirmed the ability of PKA for binding known inhibitors in our three-hybrid split-luciferase luminescence assay using staurosporine tethered to Jun. We found that loop mutants N99−F100 and R137−F138 have a 4-fold lower signal with respect to PKA-WT (2−351) while K192−G193 showed 5-fold higher signal than the wild-type (Figure 5B).

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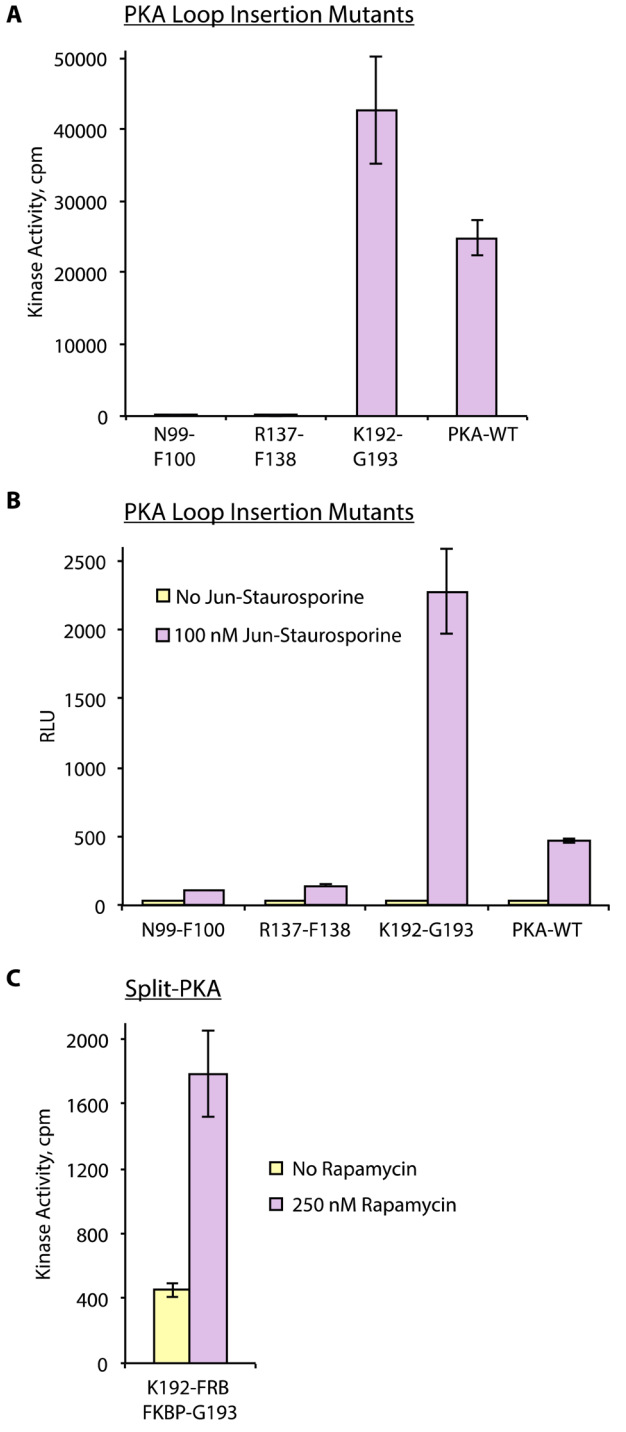

Figure 5. Split-PKA, an AGC group kinase. (A) Results from kinase assays for a 25-residue loop insertion at the positions in the catalytic domain of PKA as indicated on the *x*-axis. The insertion mutants were expressed in vitro, purified, and ^{32}P incorporation was measured with its substrates GRTGRRNSI and ^{32}P-ATP. (B) Luminescence results for Jun−staurosporine binding to loop mutants and PKA-WT in a three-hybrid split-luciferase system. (C) Activity assays for split-PKA at the indicated fragmentation site clearly demonstrate that kinase activity is recovered only in the presence of added rapamycin. Data from activity assays are not normalized and are presented after background correction of equivalent treatment of wheat germ extract.

Thus, having identified the K192−G193 site for loop insertion, we next constructed a FKBP- and FRB-dependent split-kinase, K192−FRB/FKBP−G193. As with the members of the TK group, we found that split-PKA only showed activity in the presence of 250 nM rapamycin (Figure 5C). Thus, these results provide validation that our methodology for the identification of loop insertion sites and subsequent generation of ligand-gated split-kinases may be general and applicable to kinase groups beyond TKs.

■ CONCLUSIONS

Herein we demonstrate the design, synthesis, and validation of four different ligand-activated split-kinases. We have described a potentially general step-by-step methodology for the identification of tolerant loop insertion sites within kinases based on sequence alignment, which are predictive of fragmentation sites for creating split-kinases. This is an essential first step toward the application and validation of ligand inducible split-kinases in cellulo and in vivo, which are the long-term goals. We also note that chimeras of closely related kinases have been shown to retain substrate specificity, which is largely determined by the C-terminal domain, and future experiments will probe whether the split-kinases designed herein faithfully maintain substrate specificity in cellulo.[37,38] Ultimately, these first-generation split-kinase designs must be directly compared against available methods, especially the FKBP insertional mutants developed by Hahn and co-workers. The present methodology is distinct from the FKBP-insertion approach, where significant redesign will be necessary in order to use alternate chemical inducers of dimerization (CIDs). In comparison, the split-kinase approach presented herein is modular in nature and has the potential to be amenable for controlling the activity of multiple kinases with orthogonal CIDs, which if successful will not only allow for probing the biology of signaling networks but also allow for controlling such networks, a long-term goal for synthetic biology.

Though we have utilized the classical rapamycin-induced dimerization of FKBP and FRB in our initial experiments, we anticipate that for in cellulo applications, alternate CIDs will be required that do not perturb cellular signaling, for example, designed analogs of rapamycin.[39,40] Moreover, CID systems that can be turned-on and subsequently turned-off will need to be systematically identified. It is possible that the split-kinases identified herein will be amenable for gating with light, as has been recently demonstrated for controlling protein−protein interactions with the light-dependent CRY and LOV domains.[15,41,42] The gating of kinase activity presented herein complements ongoing efforts toward understanding kinase specificity by both traditional and nontraditional approaches.[43−46] More generally, the sequence-based identification of fragmentation sites may also prove to be general and be applicable to rendering a wide range of useful proteins and enzymes under ligand inducible control, such as IR fluorescent proteins, phosphatases, or lysine deacetylases, which we are presently investigating.

■ EXPERIMENTAL SECTION

Restriction enzymes and Taq polymerase were obtained from NEB, RiboMAX large scale RNA production system was obtained from Promega. Oligonucleotide primers were obtained from IDT. Wheat germ lysate (WGL), rabbit reticulocyte lysate (RRL), and reagents for cell-free expression were obtained from Luceome Biotechnologies or Promega. Ni-NTA column was obtained from Qiagen. Protein substrate for FAK (poly(Glu:Tyr) 4:1) was obtained from Sigma Aldrich. Lyn/Src substrate (EDPIYEFLPAKKK) and PKA substrate (GRTGRRNSI) were synthesized through solid-phase peptide syn-

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thesis. Budget Solve Complete count was obtained from RPI, and ^{32}P-ATP was obtained from Perkin-Elmer. Rapamycin was obtained from LC Laboratories. Lyn-WT corresponds to residues 211−512, FAK-WT corresponds to residues 374−708, and PKA-WT corresponds to residues 2−351.

Cloning and mRNA Generation. PCR of DNA strands encoding protein fragments of interest were generated using appropriate forward and reverse primers. N-Terminal kinase PCR products were cloned using *Bam*HI/*Not*I sites into a pRSFDuet-1 vector containing a 25-residue linker flanked by *Not*I and *Mfe*I restriction sites. C-Terminal kinase PCR products were cloned using *Mfe*I/*Xho*I sites into positive clones of pRSFD-N-term kinase-linker. FKBP (residues 1−107) and FRB (residues 2025−2113 of mTOR) PCR products were cloned into the pRSFD-vector containing the linker using *Bam*HI/*Not*I and *Mfe*I/*Xho*I, respectively. N-Terminal and C-terminal products were cloned into the FKBP−linker and linker−FRB plasmids generated previously. All the positive clones were identified by dideoxyoligonucleotide sequencing. To generate mRNA, PCR fragments containing a T7 RNA polymerase promoter, a mammalian Kozak sequence, and the fragment of interest were generated. mRNA was produced according to the manufacturer's protocol. Briefly, 3 μg of PCR products were transcribed using RiboMax large scale RNA production system (T7) for 4 h at 30 °C in a final volume of 25 μL. All mRNA used was purified using G50-microcolumns.

Cell-Free Protein Expression for WT and Loop Mutants. Proteins were expressed using WGL or RRL according to standard protocols. Briefly, 1 pmol of mRNA was translated in WGL for 90 min at room temperature in a final volume of 12.5 μL. Likewise, proteins were translated for 90 min at 30 °C for RRL in a final volume of 25 μL.

Cell-Free Protein Expression for Split Proteins. One picomole of each split protein was coexpressed in WGL or RRL in the presence of 250 nM of rapamycin or 0.1% DMSO for controls as described above.

Protein Purification. Five microliters of Ni−NTA resin was equilibrated in 100 μL of wash buffer A (20 mM Tris, 250 mM NaCl, 20 mM imidazole, pH 8) for 30 min at 4 °C. The resin was spun down at 1020 rcf for 1 min and 75 μL of supernatant removed. Protein translation products were diluted to a final volume of 100 μL in buffer A and added to Ni−NTA resin, and equilibrated for 1 h at 4 °C. The resin was spun down and 100 μL of supernatant removed. The resin was washed for 4 min with 100 μL each time with the following buffers: 1× wash buffer A, 4× wash buffer B (20 mM Tris, 250 mM NaCl, 20 mM imidazole, pH 7), 1× reaction buffer (20 mM MOPS, 1 mM EDTA, 10 or 100 mM $MgCl_2$, pH 7). All buffers were supplemented with 250 nm rapamycin or 1% DMSO for purification of split-kinases.

Kinase Assays. All radioactive ^{32}P incorporation assays were carried out in duplicate and repeated on different days. Proteins were expressed in RRL or WGL separately. The assay was carried out in a final volume of 30 μL. Protein on resin was incubated with the substrate [EDPIYEFLPAKKK for Lyn and Src at 24 and 370 μM, respectively, poly(E:Y) 4:1 for FAK at 1 mg/mL, and GRTGRRNSI for PKA at 11 μM] and reaction buffer (supplemented with 250 nM rapamycin for split-kinases) for 30 min at room temperature. Ten microliters of ^{32}P-ATP mix (100 μM) was added to the protein substrate mix and incubated for 4 h at room temperature. The reaction was spotted onto P81 paper. The P81 paper was washed for 3 min three times with 500 mL of 0.85% phosphoric acid and once with 500 mL of acetone. The P81 paper was immersed in 10 mL of scintillation cocktail and the radioactive counts measured. All data are presented without normalization as total radioactive counts subtracted from reactions containing only lysate.

Split-Luciferase Assays. All luminescent assays were carried out in duplicate as described previously and repeated on different days.[31] Briefly, 0.2 pmol of Cfluc-Kinase and 0.5 pmol of Fos-Nfluc were cotranslated in RRL for 90 min at 30 °C in a final volume of 25 μL. One microliter of Jun−staurosporine or Jun−dasatinib at 2.5 μM or DMSO for negative control was added to 24 μL of translated proteins and incubated for 30 min at room temperature. Twenty microliters of complex and 80 μL of luciferase assay reagent were incubated for 1 min, and luminescence was measured using a Turner Biosystem 20/20n luminometer with 10 s integration time.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete alignments, list of constructs, controls, and experimental procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

Notes. The authors declare no competing financial interests.

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