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Direct Chemical Activation of a Rationally Engineered Signaling Enzyme

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Abstract

Few chemical strategies for activating enzymes have been developed. Here we show that a biarsenical compound (FIAsH) can directly activate a rationally engineered protein tyrosine phosphatase (Shp2 PTP) via disruption of autoinhibitory interactions between Shp2's N-terminal SH2 domain and its PTP domain. We find that introduction of a tri-cysteine motif at a loop of Shp2's N-SH2 domain confers affinity for FIAsH; binding of FIAsH to the cysteine-enriched loop relieves Shp2's inhibitory inter-domain interaction and substantially increases the enzyme's PTP activity. Activation of engineered Shp2 is substrate-independent and is observed in the contexts of both purified enzyme and complex proteomes. A chemical means for activating Shp2 may be useful for investigating its roles in signaling and oncogenesis, and the loop-targeting strategy described herein may provide a blueprint for the development of target-specific activators of other autoinhibited enzymes.

Keywords

protein engineering; hydrolases; enzyme activation; Shp2; protein tyrosine phosphatases

The vast majority of synthetic small molecules that selectively perturb enzymatic activity function as inhibitors, even though small-molecule enzyme activators can prove equally useful for interrogating the individual components of signaling networks.^[1] Compared to enzyme inhibitors, activators are much more difficult to discover, as most enzymes do not contain known activator-binding sites. The number of naturally occurring enzymes with well characterized, targetable activator-sensitive sites is very small (roughly in the teens to twenties),^[2] whereas essentially every enzyme possesses a potentially targetable site for inhibition, the active site.

One possibility for circumventing the paucity of naturally occurring enzyme-activation sites is to engineer activator-sensitivity into target proteins of interest.^[2c] Powerful and widely applicable technologies for controlling the protein-protein interactions of engineered fusion proteins with either light (optogenetics^[3]) or small molecules (chemical inducers of

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dimerization^[4]) have been developed. These approaches can afford exquisitely specific control over enzymes whose activities are dependent on specific protein-protein interactions, cellular localization, or multimerization state. Strategies for direct chemical activation (*i.e.*, not mediated through another protein) of engineered enzymes are generally less broadly applicable and often involve chemical rescue, in which introduction of an enzymatic defect into the target enzyme is coupled with administration of a small molecule that is capable of complementing the defect and restoring activity.^[5] Although rescue strategies differ quite dramatically in their chemical details, they generally share the feature of “lost” enzymatic activity in the absence of the rescuer compound—that is, an introduced mutation reduces the target enzyme’s inherent activity, which is restored (partially or fully) by a small molecule.

While chemical-rescue strategies have been important means for developing activatable enzymes, the requirement that the enzymatic activity is disrupted before it can be rescued represents a limitation for chemical biology, namely, that the small-molecule-responsive enzyme is not functional in the absence of an added small molecule. We therefore sought to investigate whether an enzyme could be engineered such that it is chemically activatable while retaining its basal activity and regulatory-control mechanism in the absence of the small-molecule activator. Tunable, small-molecule control of basal-to-activated transitions could potentially provide a means for probing enzyme function that is more physiologically relevant than chemical rescue, particularly for tightly regulated enzymes.

The activities of many signaling enzymes are regulated through protein-protein and/or inter-domain interactions. Toward the goal of engineering an activator-sensitive enzyme that retains its basal activity, we chose to target the autoinhibitory inter-domain interactions of a multi-domain signaling protein, namely, the oncogenic protein tyrosine phosphatase (PTP) Shp2.^[6] The myriad connections between Shp2 mutations and the prevalence of human pathologies such as leukemias, solid tumors, LEOPARD syndrome, and Noonan syndrome highlight Shp2’s importance as a target for study.^[7] More specifically, many disease-associated Shp2 mutants demonstrate increased basal PTP activity compared to the wild-type enzyme, yet the molecular details of the connection between misregulated Shp2 activity and pathogenesis are not well understood.^[7] We therefore set out to develop a chemically activatable Shp2 mutant that retains its basal activity and autoinhibitory control but is responsive to a small-molecule stimulus (Figure 1A).

Shp2’s phosphatase activity is controlled by an autoinhibitory intramolecular interaction between its catalytic PTP domain and its amino-terminal SH2 domain (N-SH2). In Shp2’s autoinhibited state, the N-SH2 domain blocks the PTP-domain’s active site.^[6, 8] Shp2 is activated when its N-SH2 domain binds to a suitable cellular target, transmitting a conformational change through the SH2 domain’s EF loop and thereby disrupting Shp2’s inter-domain inhibitory interaction (Figure 1B).^[6, 8] Inspired by Shp2’s cellular mechanisms of autoinhibition and activation, we hypothesized that binding of a biarsenical small molecule, FIAsh-EDT₂ (henceforth “FIAsh” for brevity),^[9] to an engineered cysteine-rich motif in the N-SH2 domain’s EF loop could induce a conformational change that would activate Shp2 by disrupting its inter-domain interaction.

Although the canonical FIAsh-binding motif contains four cysteine residues (CCXXCC), we have previously shown that potent control of enzymatic activity by biarsenical compounds can be achieved through engagement of only three cysteines.^[10] We therefore sought to minimize the perturbation of the N-SH2/PTP interface caused by the protein engineering and utilized a three-cysteine design strategy. Accordingly, we prepared two mutants of human Shp2 that each contain a tri-cysteine motif in their respective EF loops (TC: Y66C/E69C/K70C, Figure 1C): one in the context of an otherwise unmodified enzyme (TC Shp2), and one in the context of a mutation (C333P) that reduces the Shp2 PTP domain's unusual sensitivity to inhibition by FIAsh (TC/C333P Shp2). (We have previously found that, in contrast to other classical PTPs, Shp2's PTP domain can be inhibited by biarsenicals due to the presence of C333, a non-conserved cysteine residue that constitutes part of a Shp2-specific allosteric-inhibition site.^[11])

A significant concern that arises when attempting to introduce novel small-molecule sensitivity into a multi-domain interface is that the engineering itself could reduce the inherent affinity of the inter-domain interaction. In the case of an auto-inhibited enzyme, weakening of the inter-domain interaction would be expected to yield a mutant with heightened basal activity.^[12] Our design goal was to generate a Shp2 mutant that retains its inter-domain regulatory control in the absence of ligand; accordingly, we attempted to select sites within the EF loop at which cysteine mutations could be introduced without disrupting the N-SH2/PTP-domain interface. To test the effects of the enzyme engineering, we assayed the PTP activities of the wild-type and engineered enzymes with the artificial PTP substrate 6,8-difluoro-4-methylumbelliferyl phosphate (diFMUP) in the absence of FIAsh (Supporting Information, Figure S1). Although all of three of the engineered enzymes demonstrated somewhat increased activities over wild-type Shp2, the effects are modest. Moreover, introduction of the TC motif does not consistently correlate with an increase in basal activity, as shown by the indistinguishable PTP activities of C333P Shp2 and TC/C333P Shp2 (Figure S1).

We have previously shown that wild-type Shp2 is strongly inhibited by the application of FIAsh, due to the presence of a cryptic allosteric-inhibition site on the enzyme's PTP domain.^[11] We found, however, that incubation of either TC Shp2 or TC/C333P Shp2 with 1 μ M FIAsh gives rise to a measurable *increase* in PTP activity (Figure 2), suggesting that FIAsh is indeed capable of disrupting the autoinhibitory inter-domain interactions in the TC mutants in accordance with our design strategy. The activation of TC Shp2 is detectable but modest (Figure 2A), consistent with a model in which enzyme activation by FIAsh binding at the EF loop is in competition with inhibition induced by FIAsh binding at Shp2's naturally occurring PTP-domain allosteric site.^[11] By contrast, the activation of TC/C333P Shp2 is robust: a FIAsh-induced PTP-activity increase of approximately 3-fold is consistently observed over a range of substrate concentrations (Figure 2B). Based on TC Shp2's low level of activation, as well as the potential complications deriving from simultaneous activating and inactivating FIAsh-binding events on the enzyme, we focused our further efforts on TC/C333P Shp2, which contains only a single FIAsh-binding site and demonstrates strong, unambiguous FIAsh-induced activation.

To more fully characterize the nature of the N-SH2-domain/FIAsH interaction, we next investigated the dose-dependence of TC/C333P-Shp2 activation and found that significant activation of the mutant can be observed even at very low FIAsH concentrations (75 nM) that approach the concentration of enzyme (25 nM) in the assay. Half maximal activation occurred between 225 nM and 675 nM and maximal activation was reached in the low micromolar range (Figure 3A). To investigate the substrate (in)dependence of chemical activation of TC/C333P Shp2, we incubated the enzyme with FIAsH and measured its resulting PTP activity with a phosphopeptide substrate (DADEpYLIPQQG).^[13] We found that FIAsH treatment (1 μ M) leads to an approximately 2.5-fold increase of TC/C333P Shp2's initial rate of phosphopeptide dephosphorylation (Figure 3B), a degree of activation that is directly comparable to that observed with the small-molecule substrate diFMUP. The substrate-independence of activation is consistent with our engineering strategy: we would hypothesize that, as observed, release of the PTP domain from its autoinhibitory N-SH2 domain should activate the enzyme toward any potentially dephosphorylatable substrate. Taken together, our kinetic and dose-dependence data show that TC/C333P Shp2 can be directly activated with a small molecule in a manner that is potent, tunable, and substrate-independent.

We also investigated the time-dependence of Shp2 activation and found that TC/C333P Shp2 achieves measurable activation within 10 minutes of FIAsH administration, with further activation to maximal levels occurring over the course of approximately one hour (Figure 4A). While some room for improvement of the Shp2-activation kinetics remains, these data suggest that relatively short pre-incubations of FIAsH with TC-Shp2-expressing cells, lysates, or immunoprecipitates may be sufficient to induce activation in signaling experiments. Moreover, the FIAsH-induced activation of TC/C333P Shp2 is fully reversible, as demonstrated by the addition of 2,3-dimercaptopropanol (also known as British anti-Lewisite, or BAL)^[15] to pre-activated TC/C333P Shp2/FIAsH complexes (Figure 4B). "Unactivation" through addition of BAL returns the enzyme to the PTP-activity level of untreated TC/C333P Shp2 and occurs over a timeframe very similar to that observed for activation kinetics, with complete reversal of activation requiring approximately 90 minutes.

We next asked whether TC/C333P Shp2 mutants could be targeted for direct chemical activation in the context of a complex proteomic mixture. To do so, we measured the effects of FIAsH on the total PTP activities of crude lysates from TC/C333P Shp2-expressing bacteria. Because the *E. Coli* genome does not encode for PTPs, we surmised that we may be able to detect a substantial FIAsH-induced increase in Shp2 activity, even in the context of hundreds of bacterial proteins. Indeed, we find that the total activity of the TC/C333P Shp2-containing lysates increases substantially upon incubation with FIAsH (Figure 5A). The increase in crude-lysate PTP activity is dose-dependent and reaches maximal levels in the micromolar range. In the absence of the TC motif, no activation is observed; indeed wild-type Shp2 and C333P Shp2-containing lysates show partial inhibition of PTP activity (strong inhibition for wild-type Shp2, weak for C333P) when treated with FIAsH (Supporting Information, Figure S2).

Finally, to ensure that the FIAsH-induced lysate activation was attributable to a direct interaction between the small molecule FIAsH and its target TC/C333P Shp2, we performed

SDS/PAGE on FIAsh-treated lysates and visualized the FIAsh-binding proteins by fluorescence (Figure 5B and Supporting Information, Figure S3).^[9a, 16] We observed a strongly fluorescent band for TC/C333P-Shp2-expressing lysates at 68 kD (Figure 5B, lane 4), showing that FIAsh directly binds to TC/C333P Shp2 and that the resulting interaction is sufficiently strong to persist through the SDS/PAGE experiment. By contrast, the corresponding band is absent in lanes that contain lysates from cells that express Shp2 constructs lacking the TC motif, either C333P Shp2 (Figure 5B, lane 2) or wild-type Shp2 (Figure S3). Together, our lysate-activation and lysate-fluorescence data show that FIAsh is capable of directly targeting the TC motif and upregulating enzymatic activity, even in the context of complex proteomes.

In summary, we have successfully designed a rationally engineered construct of the oncogenic phosphatase Shp2 that can be target-specifically activated by a small molecule in the context of purified enzyme or a complex proteomic mixture. Compound administration induces a robust and substrate-independent increase in Shp2 activity. This engineered Shp2 enzyme, which possesses tunable activity, may provide a tool for the future *in cellulo* activation of Shp2 toward the elucidation of Shp2's signaling roles, including the poorly understood connections between Shp2 activity and the pathogenesis of cancer, Noonan, and LEOPARD syndromes. More generally, we show that targeting a biarsenical compound to a cysteine-enriched loop region of a protein's domain-domain interface can be sufficient to disrupt inter-domain interactions, thereby inducing dramatic changes in enzyme activity. The chemical tools utilized in our approach (protein-loop engineering, biarsenical compound) could, in principle, be applied in an analogous strategy to any protein target that is regulated by inter-domain interactions. Because the activities of many signaling proteins are controlled through domain-domain and/or protein-protein interactions, the Shp2-engineering strategy described here may provide a generally applicable mechanism for exerting small-molecule control over the activities of biologically important signaling proteins.

Experimental Section

General

FIAsh was synthesized as described previously.^[9a, 17] All error bars represent standard deviations from three independent experimental measurements.

Expression and purification of Shp2 enzymes

A plasmid for the expression of histidine-tagged Shp2 was a generous gift from Ben Neel and Nickolay Chirgadze (University Health Network).^[18] Site-directed mutagenesis was carried out using Quikchange according to the manufacturer's instructions. (See Supporting Information for sequences of mutagenic primers.) For protein expression, BL21(DE3)-codonPLUS *E. coli* strains containing the appropriate Shp2 plasmids were grown to midlog phase and induced with 1 mM IPTG for 16–18 hours at 16 °C. Cells were then harvested by centrifugation and stored at –80 °C. Wild-type and engineered Shp2 enzymes were purified essentially as previously reported.^[18] (See Supporting Information for details.) In experiments utilizing crude lysates, Shp2-expressing cells were grown and harvested as

described above. For lysis, cell pellets were resuspended in PTP buffer (50 mM 3,3-dimethyl glutarate pH 7.0, 50 mM NaCl, 1 mM EDTA), supplemented with TCEP (0.2 mM), Protease Inhibitor Cocktail P8849 (Sigma Aldrich), phenylmethylsulfonylfluoride, and benzamidine. Clarified lysates were divided into aliquots, flash-frozen, and stored at -80°C .

PTP-activity and FIAsH-activation assays with purified Shp2 (DiFMUP)

PTP activities of Shp2 were determined by measuring the rate of dephosphorylation of DiFMUP (Life Technologies; 7.81–125 μM). Enzymes were diluted in PTP buffer to a final assay volume of 200 μL (Shp2 concentration: 25 nM) and incubated with either DMSO (vehicle, 0.5% v/v) or FIAsH (75 nM–3 μM) at 30°C . PTP activity was then initiated with DiFMUP. The increase in fluorescence signal over time was measured using an M5 SpectraMax fluorescence reader (excitation: 360 nm, emission 450 nm). For experiments investigating the kinetics of Shp2, the rates of DiFMUP phosphorylation at various substrate concentrations were fitted to the Michaelis-Menten equation using SigmaPlot 12.3. For reversibility experiments, enzymes were first diluted in PTP buffer to a final assay volume of 200 μL (Shp2 concentration: 2.5 μM) and incubated with either DMSO (vehicle) or FIAsH (100 μM) at 30°C for 90 minutes. The mixture was then diluted 1:10 in PTP buffer to a final assay volume of 200 μL and incubated with either DMSO (vehicle) or BAL (100 μM) at 30°C . Aliquots were taken out at 45 minutes and 90 minutes and diluted 1:10 in PTP buffer to a final assay volume of 200 μL (Shp2 concentration: 25 nM) for PTP assays using DiFMUP (50 μM) as substrate.

PTP-activity and FIAsH-activation assays with purified Shp2 (phosphopeptide)

TC/C333P Shp2 was dissolved in peptide buffer (50 mM 3,3,-dimethyl glutarate pH 7.0, 125 mM NaCl, 1mM EDTA) to a final volume of 140 μL and concentration of 25 nM and incubated with 1 μM FIAsH (or DMSO) at 30°C for 90 minutes. Assays were initiated by the addition of 100 μM phosphopeptide substrate (DADEpYLIPQQG, Calbiochem) and reaction progress was monitored in a quartz micro cuvette using UV/Vis spectrophotometry at 282 nm.^[14]

PTP-activity and FIAsH-activation assays in cell lysates

Lysates from TC/C333P Shp2 Shp2-expressing cells (see above) containing 8.40 μg of total protein in PTP buffer were incubated with varying concentrations of FIAsH (1–9 μM) or DMSO at 30°C for 90 minutes. Following pre-incubation, PTP activities of the enzyme mixtures were measured with DiFMUP (50 μM).

SDS/PAGE and fluorescence visualization of FIAsH-binding proteins

Cell lysates from Shp2-expressing cells (see above) containing 16.8 μg of total protein were incubated with 10 μM FIAsH or DMSO at 30°C for 30 minutes. After pre-incubation, SDS/PAGE loading buffer (Life Technologies) was added and the samples were heated at 80°C for 10 minutes for protein denaturation. Samples were loaded into a NuPAGE Bis-Tris precast gel (Life Technologies) and run per the manufacturer's instructions. The

fluorescence of FIAsH–protein conjugates was visualized under UV light; the gel was then Coomassie stained to visualize total protein loading.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Engineering strategy for the generation of rationally designed, chemically activatable Shp2 variants. A) Binding of the small molecule (FIAH, orange circle) to a tri-cysteine (TC) motif introduced at the EF loop of Shp2's N-SH2 domain potentially shifts Shp2's conformation from a closed, low-activity state to an open, activated state. B) Structural basis for EF-loop targeting strategy. Overlay of a phosphopeptide-bound Shp2 N-SH2 domain (PDB 1AYD)^[8b] on autoinhibited Shp2 (PDB 2SHP).^[8a] Upon N-SH2/phosphopeptide binding, the domain's EF loop undergoes a conformational change (compare yellow and lavender EF loops) and the inter-domain autoinhibition is relieved. In the current study, FIAH is targeted to a TC motif at the EF loop, potentially inducing a structural change that results in chemical activation of Shp2. The EF-loop positions of the engineered cysteine residues are shown in red. The position of C333 in the PTP domain is indicated with an arrow. C) Partial primary-sequence alignment of Shp2 and the engineered Shp2 mutants used in the current study.

Figure 2.

FIAshH induces a pronounced increase in TC/C333P Shp2's PTP activity. Activities of engineered Shp2 enzymes (A, TC Shp2; B, TC/C333P Shp2) were measured with the small-molecule PTP substrate diFMUP after pre-incubation in the absence (vehicle only, light gray) and presence (1 μM , black) of FIAshH.

Figure 3.

FIAsH-induced Shp2 activation is dose-dependent and substrate-independent. A) The PTP activity of TC/C333P Shp2 was measured with DiFMUP as substrate in the presence of the indicated FIAsH concentrations after 90-minute pre-incubations. PTP activities were normalized to a vehicle-only control (100%). B) Activation of TC/C333P Shp2 using the phosphopeptide DADEpYLIPQQG as substrate. The activity of TC/C333P Shp2 was measured after pre-incubation in the absence and presence (1 μM) of FIAsH. Initial rates of phosphopeptide dephosphorylation were monitored by the increase in absorbance at 282 nm as described.^[14]

Figure 4.

FIAsH-induced Shp2 activation is time-dependent and reversible. A) The PTP activity of TC/C333P Shp2 was measured with DiFMUP as substrate in the presence of FIAsH (1 μ M) after pre-incubation for the indicated times. PTP activities were normalized as in Figure 3A. B) The PTP activity of TC/C333P Shp2 was measured after pre-incubation with FIAsH, followed by treatment with vehicle (black bars) or 100 μ M 2,3-dimercaptopropanol (BAL, gray bars) for the indicated times. PTP activities were normalized to double-vehicle controls (DMSO added in lieu of both FIAsH and BAL).

Figure 5.

FIAsH targets and activates engineered TC/C333P Shp2 in a complex proteome. A) Target-specific activation of TC-Shp2 mutants in *E. Coli* lysates. Crude bacterial lysates were treated with the indicated FIAsH concentrations and total PTP activities of the lysates were measured with DiFMUP (50 μM) as substrate. PTP activities were normalized to a DMSO-only (100%) control. B) Fluorescence visualization of a TC/C333P-FIAsH adduct. Lysates from cells that expressed the indicated Shp2 constructs were treated with DMSO (–) or 10 μM FIAsH (+). After the proteins were separated by SDS/PAGE, FIAsH-bound proteins were visualized under UV light. The Shp2 molecular weight is indicated by arrow. For a protein-loading control all proteins were visualized by Coomassie staining (Supporting Information, Figure S3).