



Target-specific control of lymphoid-specific protein tyrosine phosphatase (Lyp) activity

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ABSTRACT

Lymphoid-specific protein tyrosine phosphatase (Lyp), a member of the protein tyrosine phosphatase (PTP) superfamily of enzymes, is an important mediator of human-leukocyte signaling. Lyp has also emerged as a potential anti-autoimmune therapeutic target, owing to the association of a Lyp-activating mutation with an array of autoimmune disorders. Toward the goal of generating a selective inhibitor of Lyp activity that could be used for investigating Lyp's roles in cell signaling and autoimmune-disease progression, here we report that Lyp's PTP domain can be readily sensitized to target-specific inhibition by a cell-permeable small molecule. Insertion of a tetracysteine-motif-containing peptide at a conserved position in Lyp's catalytic domain generated a mutant enzyme (Lyp-CCPGCC) that retains activity comparable to that of wild-type Lyp in the absence of added ligand. Upon addition of a tetracysteine-targeting biarsenical compound (FlAsH), however, the activity of the Lyp-CCPGCC drops dramatically, as assayed with either small-molecule or phosphorylated-peptide PTP substrates. We show that FlAsH-induced Lyp-CCPGCC inhibition is potent, specific, rapid, and independent of the nature of the PTP substrate used in the inhibition assay. Moreover, we show that FlAsH can be used to specifically target overexpressed Lyp-CCPGCC in a complex proteomic mixture. Since the mammalian-cell permeability of FlAsH is well established, it is likely that FlAsH-mediated inhibition of Lyp-CCPGCC will be useful for specifically targeting Lyp activity in engineered leukocytes and autoimmune-disease models.

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1. Introduction

Protein tyrosine phosphatases (PTPs) catalyze the dephosphorylation of phosphotyrosine, a central signal-transduction control element in metazoan biology.¹ Improperly regulated PTP activity has been implicated as a causative agent in a range of human diseases, including leukemia, solid-tumor cancers, diabetes, and autoimmune disorders.^{2–6} While many disease-associated mutations in PTP-encoding genes are loss-of-function mutations, *gain-of-function* PTP mutations are of particular interest from a therapeutic perspective; it is this relatively small subset of PTP disease-associated mutants that one could envision as therapeutic targets for small-molecule PTP inhibitors. Among the best characterized of PTPs that have been linked to activating disease-associated mutations is the lymphoid-specific protein tyrosine phosphatase (Lyp, also called PTPN22), which is expressed predominantly in leukocytes and is a negative regulator of T-cell activation.⁷ Recently, a flurry of studies has uncovered associations between a single-nucleotide polymorphism (SNP) in the *PTPN22* gene and a range of autoimmune disorders, including type-I diabetes,^{8,9} rheumatoid

arthritis,¹⁰ Graves disease,¹¹ myasthenia gravis,¹² and systemic lupus erythematosus (SLE).¹³ The SNP common to all of these associations encodes an arginine-to-tryptophan mutation (R620W) that increases Lyp's PTP activity.¹⁴ This putative connection between high Lyp activity and autoimmune disease has become even more compelling with the recent discovery of a PTP-activity-lowering polymorphism (R263Q) that confers *protection* against SLE.¹⁵ Taken together, these studies present the exciting possibility that Lyp inhibitors could represent an important class of anti-autoimmune therapeutics. Moreover, these genetic data highlight the need for chemical tools that can be used to study the poorly understood connection between Lyp activity and autoimmune-disease progression.

PTP-inhibitor discovery is inherently difficult due to two recurring problems observed with many active-site-directed inhibitors: lack of target specificity (classical PTP catalytic domains share a significant degree of sequence and structural homology with one another) and poor bioavailability (many PTP-binding pharmacophores contain negatively charged phosphotyrosine mimetics that lower an inhibitor's cellular permeability).^{6,16–18} Nevertheless, several groups have recognized the potential therapeutic impact of target-specific Lyp inhibitors, and significant efforts toward a Lyp-specific inhibitor have recently been undertaken.^{19–23} While

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the compounds that have been identified from these studies provide important templates for further optimization and discovery. Lyp-inhibitor discovery is a newly emerging field, and chemical tools that can control Lyp activity in cells with high potency and target selectivity are still needed.

Our lab has recently described a systematic strategy for engineering novel inhibitor sensitivity in PTPs.^{24–28} In our approach, a mutation (point mutation and/or peptide insertion) in a target PTP sensitizes the enzyme to inhibition by a small molecule that does not inhibit wild-type PTPs. When a non-deleterious, inhibitor-sensitizing mutation is discovered, the mutant and inhibitor constitute a specific ligand/receptor pair that can be used to study the cellular roles of the engineered—but functionally ‘wild-type-like’—PTP targets. Toward this end, we have previously reported that PTP domains can be sensitized to noncompetitive inhibition by a compound that has no significant affinity for wild-type PTPs²⁶—namely, FIAsh, a cell-permeable biarsenical compound that binds to cysteine-rich peptides.^{29,30} Specifically, insertion of a FIAsh-binding hexapeptide (CCPGCC) at position 187 of a model PTP (TCPTP) was shown to confer strong FIAsh sensitivity on the enzyme.²⁶ Although the 187 insertion position is distal from TCPTP’s active site, the insertion mutant’s novel inhibitor sensitivity could be structurally rationalized,³¹ as Glu187 lies at the end of a conserved PTP loop (the WPD loop) that closes upon substrate binding, properly positioning a mechanistically important aspartate residue (the ‘D’ of WPD) for the PTP reaction. Binding of FIAsh to the TCPTP insertion mutant may impede proper closure of the WPD loop, in a manner that is consistent with other noncompetitive inhibitors that target natural allosteric sites in PTPs.^{32,33}

The fundamental nature of the WPD loop in the structure and mechanism of PTP domains augurs well for the prospect of using FIAsh as an inhibitor of engineered PTPs beyond TCPTP: the WPD loop is one of the most conserved regions in PTP catalytic domains and it can be readily identified from primary-sequence alignments.^{34,35} Indeed, we have recently shown that PTPs from six distinct PTP sub-families can be sensitized to FIAsh inhibition.²⁷ In all cases, the engineered PTPs respond to FIAsh with high selectivity and potency via a conserved mechanism that is independent of the particular PTP that has been sensitized to inhibition.²⁷

A key advantage of inhibitor-design strategies that exploit similarities in protein-family members (in this case, the conserved WPD loop of the PTP domain) is that successful engineering can potentially be applied to any protein-family member,³⁶ and new targets can be selected as complementary biological data reveal their importance. The autoimmune-association studies noted above have brought the need for Lyp inhibitors to the fore, and primary-sequence analysis suggests that Lyp may be amenable to FIAsh-sensitization.³⁴ Here we describe the generation and characterization of a sensitized mutant of Lyp, which will provide a novel tool for the elucidation of Lyp’s functions in engineered cells and, potentially, for the pharmacological validation of Lyp as an anti-autoimmune drug target.

2. Materials and methods

2.1. General

FIAsh was synthesized as described previously.^{26,29,37} Absorbance measurements were made on a Molecular Devices Versamax 96-well plate reader. Fluorescence measurements were made on either an ISS K2 multi-frequency phase fluorometer or a Molecular Devices Spectra Max M5 96-well fluorescence plate reader. Gel imaging and analyses were performed on a Syngene InGenius gel-documentation system. Curve fitting was carried out with SigmaPlot 11.0. Error bars and \pm values in all figures and tables

represent the standard deviations of at least three independent trials.

2.2. Cloning and mutagenesis

A PCR product encoding the Lyp catalytic domain (residues 1–294) was amplified from full-length *PTPN22* cDNA (Open Biosystems, Cat#: IHS1382-8404627) with *PfuTurbo* DNA polymerase (Stratagene) using the following primers (5′ to 3′): ATCCTGAATTC CATGGACCAAGAGAAATTCTGCAGAAAG and ATCCTAAGCTTCATCT GTCTCTTAAATAGTTCTAATACAGC. The PCR product and pET-21b were doubly digested with *EcoRI* and *HindIII*, gel purified, and ligated using T4 DNA ligase, yielding the Lyp-expressing plasmid pZEWO01. Insertional mutagenesis was carried out by QuikChange™ essentially as described for TCPTP²⁶ with pZEWO01 as template and the following mutagenic primers (5′ to 3′): GAC CATGATGTACCTTGCTGCCGGGCTGCTCATCTATAGACCCT and AGGGTCTATAGATGAGCAGCAGCCCGGGCAGCAAGGTACATCATGGTC, yielding the Lyp-CCPGCC-encoding plasmid pZEWO14.

2.3. Lyp expression and purification

BL21(DE3)-codonPLUS-RIL *Escherichia coli* (Stratagene) containing either pZEWO01 or pZEWO14 were grown overnight at 37 °C in LB. Cultures were diluted, grown to mid-log phase ($OD_{600} = 0.5$), induced with 0.2 mM isopropyl-1-thio- β -D-galactopyranoside, and shaken at 23 °C for 20 h. The cells were harvested by centrifugation, resuspended in binding buffer (50 mM Tris pH 7.9, 500 mM NaCl, 5 mM Imidazole), and lysed by French Press at ~2000 psi. Lysates were clarified by centrifugation, and enzyme purifications were carried out using SwellGel Nickel Chelated Discs (Pierce) according to the manufacturer’s instructions. The protein solutions obtained were exchanged into storage buffer (50 mM 3,3-dimethylglutarate pH 7.0, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol), concentrated, flash-frozen in liquid nitrogen, and stored at –80 °C. SDS-PAGE was used to estimate enzyme concentrations, by comparison of pixel counts of the major 37-kD band in the enzyme preparation to those of a reference protein (BSA) run on the same gel. Inspection of SDS-PAGE gels yielded estimates of >90% and >50% purity for Lyp and Lyp-CCPGCC, respectively. Nickel-affinity-chromatography-purified yields of both enzymes were approximately 2 mg/L of culture.

2.4. PTP activity and inhibition assays (pNPP substrate)

2.4.1. Michaelis–Menten kinetic assays

Activity assays on *para*-nitrophenyl phosphate (pNPP) were carried out at 25 °C in low protein-binding tubes. Solutions of wild-type Lyp or Lyp-CCPGCC (2.5 μ M) Lyp were incubated in 1 \times PTP buffer (50 mM 3,3-dimethylglutarate pH 7.0, 1 mM EDTA, 50 mM NaCl) for 2.5 h in the presence of 10 μ M FIAsh or DMSO vehicle. Solutions were then diluted in 1 \times PTP buffer, and PTP reactions were initiated by the addition of 20 μ L pNPP (varying concentrations) to 180 μ L of the enzyme solution (enzyme concentration in assay: 500 nM). Reactions were quenched by the addition of 40 μ L 5 M NaOH. 200 μ L of reaction mixture were then loaded onto a 96-well plate and the absorbance at 405 nm was measured. Kinetic constants were determined by fitting the data to the Michaelis–Menten equation.

2.4.2. FIAsh-concentration-dependence inhibition assays

FIAsh-concentration-dependence assays were carried out at 9 mM pNPP, 250 nM Lyp or Lyp-CCPGCC, and varying concentrations of FIAsh in 1 \times PTP buffer. After a 2 h incubation of enzyme and FIAsh (or vehicle control), pNPP was added. The reactions were quenched and quantified as described above.

2.4.3. Kinetics of FIAsH inhibition

Reactions were carried out in a total volume of 200 μL at 25 $^{\circ}\text{C}$. 30 μL of premixed solutions of FIAsH and pNPP were aliquoted onto a 96-well plate. 170 μL of an enzyme and buffer solution were added such that the final concentrations were 250 nM enzyme, 10 mM pNPP, and 0.5–5 μM FIAsH in $1 \times$ PTP buffer. The increase in absorbance at 405 nm was monitored with readings every 10 s for 45 min. Reaction velocity (A_t) for each dosage of FIAsH was then determined at each time point, t_x , by calculating the slope of the linear regression over the 1-min interval $t_x - 30 \leq t_x \leq t_x + 30$. Relative activity (A_t/A_0) was obtained by dividing the reaction velocity in the presence of each FIAsH dosage by the no-FIAsH control. The pseudo-first-order rate constant (k_{obs}) was determined for each FIAsH concentration by fitting the data to Eq. 1,³⁸ in which A_0 and A_{∞} represent the maximum and minimum reaction velocities, respectively:

$$\frac{A_t}{A_0} = \frac{A_{\infty}}{A_0} + \frac{A_0 - A_{\infty}}{A_0} e^{-k_{\text{obs}} \cdot t} \quad (1)$$

Each k_{obs} value was plotted against FIAsH concentration and the data were fit to Eq. 2,³⁸ in which K_i is the apparent inhibition constant, k_i is the inactivation rate constant, and $[F]$ is the concentration of FIAsH:

$$k_{\text{obs}} = \frac{k_i x [F]}{K_i + [F]} \quad (2)$$

2.5. PTP activity and inhibition assays (DADEpYLIPQQG substrate)

The PTP activities of Lyp and Lyp-CCPGCC on the phosphopeptide DADEpYLIPQQG were measured by monitoring the time-dependent increase of the peptide's fluorescence at 305 nm, essentially as described.³⁹ Briefly, solutions of 59 nM enzyme and 294 nM FIAsH (or DMSO vehicle only) were incubated for 1.5 h in peptide buffer (50 mM 3,3-dimethylglutarate pH 7, 125 mM NaCl, 1 mM EDTA). PTP reactions were initiated by the addition of DAD-EpYLIPQQG (EMD) to the enzyme-FIAsH solution such that the final reaction concentrations were 250 nM FIAsH (or control), 50 nM enzyme, and 15 μM peptide. The increase in fluorescence at 305 nm was then measured ($\lambda_{\text{exc}} = 280$ nm, $\lambda_{\text{em}} = 305$ nm, 0.5 nm slit widths) for 20 min or until substrate was consumed. To extract kinetic parameters, the intensity of fluorescence was converted to product concentration ($[p]$) for each time point (t) and fit to Eq. 3,³⁹ in which $[Lyp]$ is the total concentration of enzyme in the assay (50 nM) and $[p]_{\infty}$ is the final concentration of product (15 μM):

$$t = \frac{[p]}{k_{\text{cat}}[Lyp]} + \frac{K_m}{k_{\text{cat}}[Lyp]} \ln \left(\frac{[p]_{\infty}}{[p]_{\infty} - [p]} \right) \quad (3)$$

2.6. Fluorescence

2.6.1. Kinetics of Lyp-CCPGCC-induced FIAsH fluorescence

Wild-type Lyp or Lyp-CCPGCC (250 nM in $1 \times$ PTP buffer) was mixed with FIAsH (500 nM) in $1 \times$ PTP buffer, and FIAsH-fluorescence values (excitation: 510 nm, emission: 529 nm) of the resulting solutions were measured every 10 s. The displayed data sets were normalized by the subtraction of a FIAsH-only (no protein) control monitored over the same time range.

2.6.2. Measurement of the Lyp-CCPGCC/FIAsH apparent dissociation constant ($K_{\text{Dapp}}^{\text{app}}$)

Solutions of 25 nM FIAsH and Lyp-CCPGCC ranging in concentration from 39–2500 nM were incubated in $1 \times$ PTP buffer for

2.5 h at room temperature. The FIAsH-fluorescence values (excitation: 510 nm, emission: 540 nm) of the solutions were measured in 96-well plates, corrected by subtraction of the fluorescence from a FIAsH-only (no-protein) control, and normalized to estimate the percentage of ligand complexed to protein at each Lyp-CCPGCC concentration (ϕ). To estimate the apparent dissociation constant of the Lyp-CCPGCC/FIAsH interaction, the data were fit to Eq. 4, which is derived from first principles for a reversible, tight-binding inhibitor:^{40,41}

$$\phi = \frac{1}{2[F]} * ([F] + [Lyp] + K_{\text{Dapp}}) - \sqrt{([F] + [Lyp] + K_{\text{Dapp}})^2 - (4[F][Lyp])} \quad (4)$$

$[F]$ and $[Lyp]$ represent the total concentrations of FIAsH and enzyme in the assay, respectively.

2.6.3. Fluorescence of Lyp-expressing cells

Pellets of *E. coli* cells expressing either Lyp or Lyp-CCPGCC were prepared as described above (Section 2.3) and frozen at -80°C . Pellets from 15 mL of culture were resuspended and diluted to $\text{OD}_{600} = 6.9$ (1 cm path length) in $1 \times$ PTP buffer containing 10 μM FIAsH. After 2.5 h at room temperature, the FIAsH-fluorescence values (excitation: 510 nm, emission: 540 nm) of the suspensions were measured in 96-well plates and corrected by subtraction of the fluorescence from a FIAsH-only (no-cell) control.

2.6.4. In-gel detection of Lyp-CCPGCC/FIAsH fluorescence

Pellets of *E. coli* cells expressing either Lyp or Lyp-CCPGCC were freeze-thawed and FIAsH-treated (10 μM) as described above (Section 2.6.3). After 2.5 h at room temperature, the cell suspensions were mixed 3:1 with $4 \times$ loading buffer ($4 \times$ LDS, Invitrogen), boiled for 10 min, and loaded on a 4–12% Bis-Tris SDS-PAGE gel (Invitrogen). Following electrophoresis, fluorescent bands were visualized on a gel-documentation system using a UV transilluminator for excitation and a 500–600 nm emission filter.

3. Results and discussion

3.1. Design of and characterization of a FIAsH-sensitized Lyp mutant

Lyp is a class-I cysteine-based, classical cytoplasmic PTP.³⁴ In addition to a canonical WPD loop, Lyp possesses significant catalytic-domain homology to both TCPTP and the other PTPs to which FIAsH sensitization has been applied.^{19,35} To design a potentially FIAsH-sensitized version of Lyp we aligned its primary-sequence and catalytic-domain structure with TCPTP, the model PTP on which our PTP-sensitization was developed (Fig. 1A). Both sequence comparison and inspection of Lyp's three-dimensional structure (Fig. 1B) suggest that a CCPGCC insertion analogous to the one that has been successful for sensitizing TCPTP may work for the Lyp target as well. To test this idea, we generated the putatively FIAsH-sensitized insertion mutant Lyp-CCPGCC (Fig. 1A) by insertional mutagenesis and expressed it from *E. coli* as a six-histidine tagged protein.

For a mutant enzyme/inhibitor pair to be useful in chemical biology it is imperative that the mutated protein retains activity in the absence of the drug.^{25,42} To investigate the possible effect of the CCPGCC mutation on Lyp's PTP activity, we determined the Michaelis-Menten kinetic parameters for wild-type and Lyp-CCPGCC with the artificial PTP substrate *para*-nitrophenyl phosphate (pNPP). We found that the insertion mutant retains almost full catalytic activity in the absence of FIAsH, with a catalytic rate-constant value (k_{cat}) that is within a factor of two of the

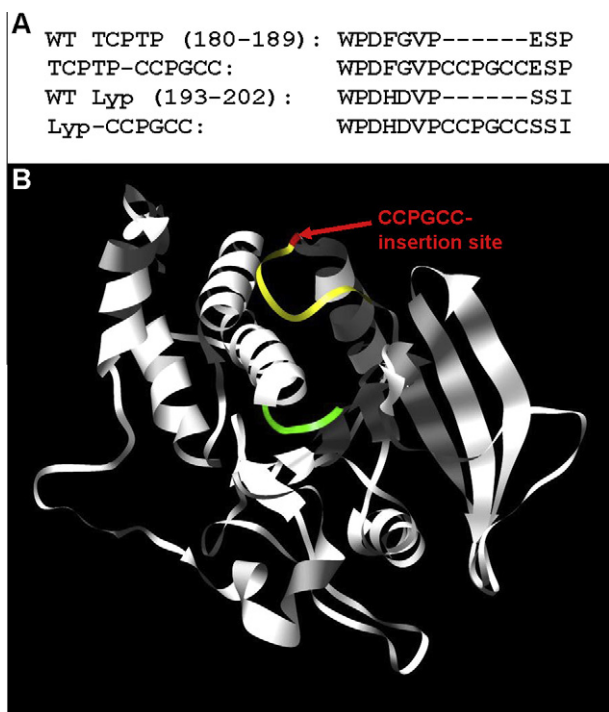


Figure 1. Design of a FIAsH-sensitized Lyp mutant. (A) Amino-acid sequence alignment of the WPD-loop region of a previously FIAsH-sensitized PTP, TCPTP (wild-type: WT, sensitized: TCPTP-CCPGCC), with that of wild-type Lyp and Lyp-CCPGCC. (B) Ribbon diagram of the Lyp catalytic domain (PDB code: 2QCJ¹⁹). Lyp's canonical WPD loop is shown in yellow with the position of the Lyp-CCPGCC insertion shown in red. For perspective, the phosphotyrosine-binding loop of the PTP active site is shown in green.

corresponding wild-type construct (Table 1). (The wild-type Lyp catalytic domain has been enzymatically characterized by others with *p*NPP previously,¹⁹ and our construct yielded kinetic constants that were in good agreement with literature data.) The tolerance of Lyp to the six-amino-acid insertion, indicated by its relatively low 1.7-fold reduction in k_{cat} , is interesting in light of the fact that Lyp's closest homolog, PEST, was substantially more affected by the analogous CCGGCC insertion (wild-type PEST: $k_{cat} = 0.59 \text{ s}^{-1}$; PEST-CCPGCC: $k_{cat} = 0.081 \text{ s}^{-1}$).²⁷ These findings suggest that primary-sequence alignments alone will not constitute a suitable guide for prediction of which PTPs are most amenable to FIAsH sensitization via CCGGCC insertion.

To further test the suitability of Lyp-CCPGCC as an inhibitor-sensitized PTP, we measured its activity with a more physiologically relevant substrate, the phosphopeptide DADEpYLIPQQG, which corresponds to the auto-phosphorylation site of the epidermal growth factor receptor (EGFR_{988–998}). With the phosphopeptide substrate we again found that, in the absence of FIAsH, Lyp-CCPGCC has only a slightly lower activity than wild-type Lyp (virtually identical 1.8- and 1.7-fold drops in k_{cat} relative to wild-type Lyp on peptide and *p*NPP, respectively; see Table 1). These data show that the catalytic activity of the CCGGCC mutant is 'wild-type-like' in activity independent of substrate and suggest that, absent FIAsH, Lyp-

CCPGCC will be capable of dephosphorylating the tyrosine-phosphorylated substrates that Lyp encounters in a mammalian cell.

3.2. Target-specific inhibition of Lyp-CCPGCC by FIAsH

We next investigated the effects of FIAsH treatment on wild-type Lyp and Lyp-CCPGCC. In previous studies from our lab, FIAsH was found to have no discernible effect upon a panel of wild-type PTPs.²⁷ Consistent with this precedent, the kinetic parameters of wild-type Lyp activity with *p*NPP were indistinguishable in the presence and absence of FIAsH (Table 2, Fig. 2A). By contrast, Lyp-CCPGCC is strongly inhibited by FIAsH: upon incubation with a fourfold molar excess of FIAsH, the specificity constant of Lyp-CCPGCC activity (k_{cat}/K_M) dropped 4.5-fold (Table 2, Fig. 2B). As previously observed with other FIAsH-sensitized PTPs, Lyp-CCPGCC inhibition appears to be noncompetitive in nature, as the loss of catalytic efficiency is due completely to a decrease in k_{cat} (Table 2).

To test whether the degree of Lyp-CCPGCC inhibition was dependent on the FIAsH dosage, we performed a concentration-dependence inhibition assay (Fig. 2C). We observed that the degree of inhibition by FIAsH is highly concentration-dependent, with 50% inhibition (IC_{50}) achieved at approximately 625 nM FIAsH. It should be noted that FIAsH so potently inhibits Lyp-CCPGCC that the IC_{50} value approaches the concentration of enzyme in the assay (250 nM Lyp-CCPGCC, the lowest concentration that can be reasonably used in the *p*NPP-based assay). Under conditions of such potent, almost stoichiometric, inhibition, measured IC_{50} values are highly dependent on the enzyme concentration used, and, therefore, of little fundamental significance. For example, under the conditions of the inhibition experiment shown in Fig. 2C (250 nM Lyp-CCPGCC), it is theoretically impossible that an inhibitor which binds enzyme in a 1:1 manner could demonstrate a 50% inhibition value of less than 125 nM, half the enzyme concentration. As described below (Section 3.4), more sensitive fluorescence-based assays reveal a substantially tighter Lyp-CCPGCC/FIAsH interaction than would be inferred from the inhibition data.

If Lyp-CCPGCC inhibition is to be useful in a cellular context, it is important that FIAsH, the target-specific inhibitor of Lyp-CCPGCC, acts in a substrate-independent manner. To ensure that the FIAsH sensitivity observed with *p*NPP was not substrate-dependent we investigated Lyp-CCPGCC's FIAsH sensitivity with DADEpYLIPQQG. Indeed, the engineered FIAsH sensitivity of Lyp-CCPGCC proved to be substrate-independent (Table 2 and Fig. 3). When incubated with FIAsH, the activity of the mutant dropped dramatically and specifically (as with *p*NPP, FIAsH had no significant effect upon wild-type). The FIAsH-induced inhibition of DADEpYLIPQQG dephosphorylation is essentially complete: following incubation with a fivefold molar excess of FIAsH, the magnitude of Lyp-CCPGCC inhibition is so great that the data could not be fit to derive kinetic constants (Fig. 3B). Thus, FIAsH-induced inhibition of peptide dephosphorylation by Lyp-CCPGCC is consistent with the potency observed with *p*NPP and suggests that FIAsH-inhibition could be effective in cellulo, where PTP substrates are more chemically similar to DADEpYLIPQQG than to *p*NPP. And, taken together, these data showing the potency, specificity, and substrate independence of Lyp-CCPGCC

Table 1
Inherent PTP activities of wild-type Lyp and Lyp-CCPGCC catalytic domains

Enzyme	k_{cat}^a (s^{-1})	K_M^a (mM)	k_{cat}^b (s^{-1})	K_M^b (μ M)
Wild-type Lyp	0.49 ± 0.04	4.86 ± 0.39	4.16 ± 0.56	8.12 ± 2.63
Lyp-CCPGCC	0.27 ± 0.02	5.05 ± 0.35	2.25 ± 0.56	17.8 ± 1.8

^a With *p*NPP used as the PTP substrate.

^b With DADEpYLIPQQG used as the PTP substrate.

Table 2

PTP activities of wild-type Lyp and Lyp-CCPGCC catalytic domains after pre-incubation with FIAsH

Enzyme	k_{cat}^a (s^{-1})	K_M^a (mM)	k_{cat}^b (s^{-1})	K_M^b (μ M)
Wild-type Lyp	0.49 ± 0.02	4.92 ± 0.35	3.84 ± 1.11	7.55 ± 4.48
Lyp-CCPGCC	0.057 ± 0.004	4.83 ± 0.44	ND ^c	ND ^c

^a With *p*NPP used as the PTP substrate.

^b With DADEpYLIPQQG used as the PTP substrate.

^c ND: unable to determine kinetic parameters due to low activity; see Figure 3B.

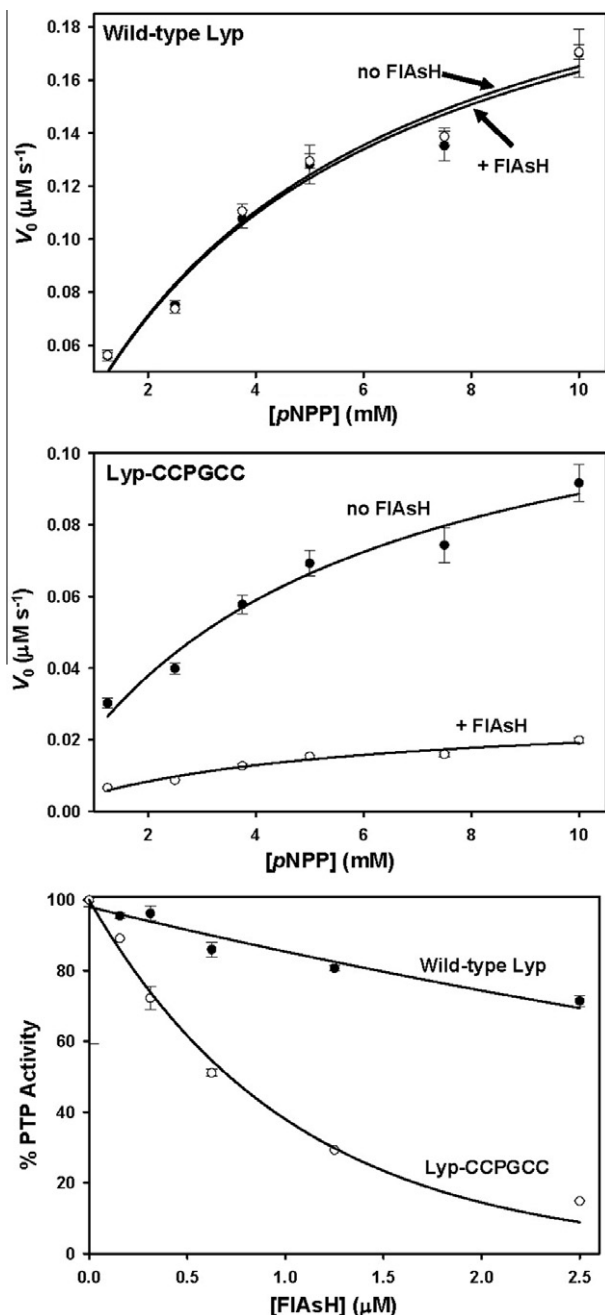


Figure 2. Target-specific inhibition of Lyp-CCPGCC. (A) WT Lyp (2.5 μM) or (B) Lyp-CCPGCC (2.5 μM) was incubated in the absence (closed circles) or presence of FIAsh (10 μM , open circles), diluted, and assayed for activity with the PTP substrate pNPP at the indicated concentrations. (C) Concentration-dependence of FIAsh-induced inhibition: wild-type Lyp (250 nM, closed circles) or Lyp-CCPGCC (250 nM, open circles) was incubated in the absence or presence (indicated concentrations) of FIAsh. The resulting solutions were assayed for activity with pNPP (9 mM). '% PTP Activity' is defined as the initial velocity in the presence of FIAsh divided by the initial velocity of the vehicle-only (100%) control.

inhibition imply that FIAsh could be used in a cellular context to target Lyp-CCPGCC without substantial off-target PTP inhibition.

3.3. Kinetics of Lyp-CCPGCC inhibition by FIAsh

In all of the inhibition experiments described above, Lyp-CCPGCC was allowed to pre-incubate with FIAsh prior to the measurement of PTP activity. Because FIAsh is not a rapid, reversible protein binder—biarsenicals make covalent sulfur–arsenic bonds with their peptide targets^{29,43}—inhibition of Lyp-CCPGCC is presumably time-depen-

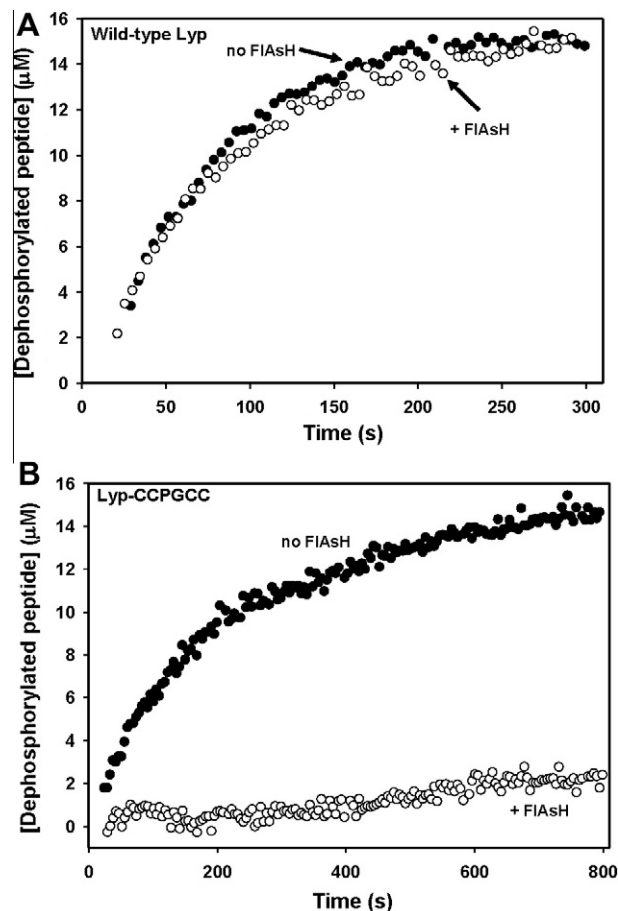


Figure 3. Target-specific inhibition of DADEpYLIPQQG dephosphorylation. (A) Wild-type Lyp (59 nM) or (B) Lyp-CCPGCC (59 nM) was incubated in the absence (closed circles) or presence of FIAsh (294 nM, open circles). After 90 min, DADEpYLIPQQG (15 μM) was added to the enzyme solutions and peptide dephosphorylation was monitored by fluorescence.

dent, and the timescale of FIAsh action on Lyp-CCPGCC is a potentially complicating issue when using the compound for controlling protein function.

To assess the rate of Lyp-CCPGCC inhibition by FIAsh, we used a continuous assay of pNPP hydrolysis to probe enzyme activity and time-dependent inhibition concurrently.⁴⁴ We continuously monitored the PTP reaction at various concentrations of FIAsh and determined that the slopes of the resulting progress curves decreased over time, indicating a time-dependent drop in the concentration of catalytically active Lyp-CCPGCC. The rate at which these progress curves fell was dependent on the concentration of FIAsh, and the exponential decay of relative activity suggested a first-order dependence on time (Fig. 4A). Analysis of the resulting pseudo-first-order rate constants (k_{obs}) as a function of FIAsh concentration showed that Lyp-CCPGCC inhibition displays saturation kinetics (Fig. 4B). The nonlinear relationship between k_{obs} and inhibitor concentration suggests a two-step model of inhibition in which FIAsh and Lyp-CCPGCC first associate in a loose, reversible manner; then, in a second step, undergo a slow conversion to an irreversibly inhibited form. Fitting the inhibition data to this model (see Section 2.4.3) yielded values for the apparent inhibition constant (K_i^{app}) of $3.04 \pm 1.05 \mu\text{M}$ and an inactivation rate constant (k_i) of $0.0067 \pm 0.0022 \text{ s}^{-1}$. These derived measures of inactivation kinetics compare favorably with other 'slow-binding' PTP inhibitors; for example, a recently reported class of aryl vinyl sulfones and sulfonates inactivate PTP domains with K_i and k_i values that are roughly 100-fold higher and fivefold lower, respectively.³⁸

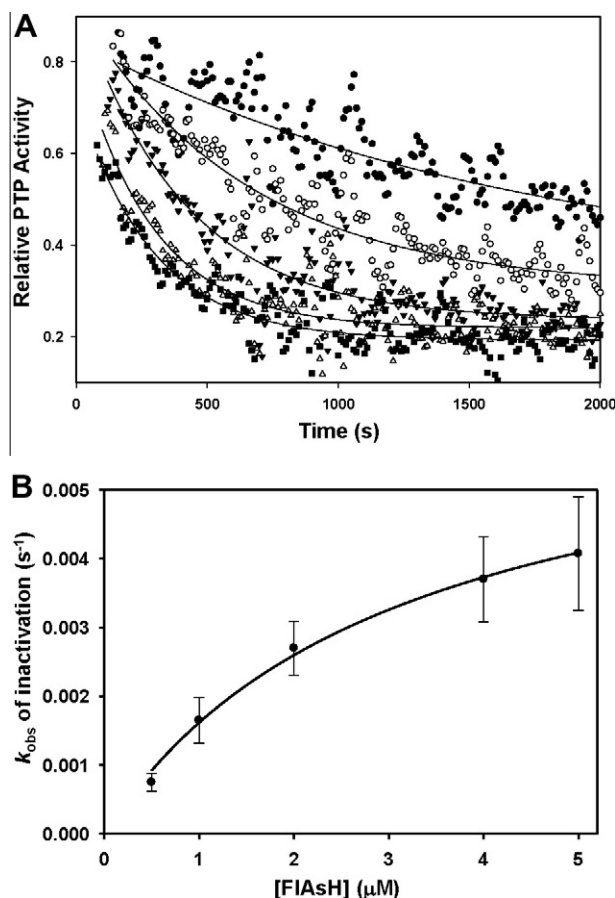


Figure 4. Kinetics of FIAsH-induced Lyp-CCPGCC inhibition. (A) Solutions of Lyp-CCPGCC (250 nM) and pNPP (10 mM) were combined with varying concentrations of FIAsH (closed circles: 0.5 μM , open circles: 1.0 μM , closed triangles: 2.0 μM , open triangles: 4.0 μM , squares: 5.0 μM) and assayed for PTP activity by continuous measurement of absorbance at 405 nm. Relative activity at each time point was computed as the slope of the reaction curve in the presence of FIAsH divided by the slope of the reaction curve for a no-FIAsH control. The curves shown derive from the fitting of the individual time points to a model of exponential activity decay with a first-order dependence on time. (B) Pseudo-first-order rate constants were derived from the curve fitting described above and plotted against the relevant indicated concentrations of FIAsH.

3.4. Fluorescence characterization of Lyp-CCPGCC/FIAsH binding

FIAsH was developed by Tsien and co-workers as a fluorescence-based protein-visualization tool, and binding of FIAsH to its tetracycline target sequence (either in the context of a short peptide or a protein) gives rise to a dramatic increase in the compound's fluorescence.^{29,30} While our primary interest lies in control of Lyp activity, not in Lyp visualization, the putative increase in FIAsH fluorescence upon Lyp-CCPGCC binding potentially provides a useful tool for characterizing the kinetics and thermodynamics of FIAsH's interactions with the target enzyme. Indeed, we found that incubation of Lyp-CCPGCC with FIAsH leads to a rapid increase in fluorescence over the course of about 10 min (Fig. 5A). By contrast, no substantial fluorescence increase was observed upon incubation of wild-type Lyp with FIAsH (Fig. 5A). These data suggest that FIAsH fluorescence can be a useful tool for specifically detecting the Lyp-CCPGCC/FIAsH interaction, either for *in vitro* biophysical studies, or in complex proteomic or cellular mixtures.

We used the Lyp-CCPGCC-induced increase in FIAsH fluorescence to estimate the apparent binding constant of the Lyp-CCPGCC/FIAsH interaction with greater sensitivity than could be achieved in pNPP-based inhibition assays. (As noted earlier, the high potency of FIAsH

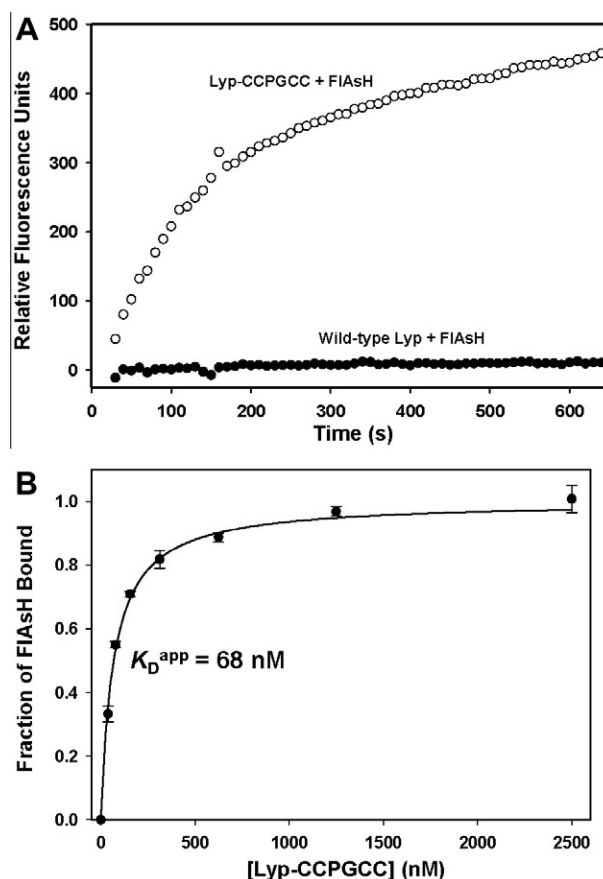


Figure 5. Lyp-CCPGCC-induced FIAsH fluorescence. (A) Wild-type Lyp (250 nM, closed circles) or Lyp-CCPGCC (250 nM, open circles) was mixed with FIAsH (500 nM), and FIAsH fluorescence was monitored over time. The displayed data sets were normalized by the subtraction of a FIAsH-only (no protein) control. (B) Determination of the Lyp-CCPGCC/FIAsH apparent dissociation constant: FIAsH (25 nM) was incubated with the indicated concentrations of Lyp-CCPGCC for 2.5 h and the FIAsH fluorescence of the resulting solutions was measured.

complicates the extraction of true inhibition-constant values from percent-inhibition values.) To estimate the apparent dissociation constant in an assay that was not constrained by detection of Lyp-CCPGCC activity, we incubated a constant amount of FIAsH (25 nM) with varying amounts of Lyp-CCPGCC and measured the endpoint FIAsH-fluorescence values of the resulting solutions. As shown in Fig. 5B, even at very low Lyp-CCPGCC concentrations FIAsH is largely protein-bound, and a binding-model fit of the fluorescence data yields an apparent dissociation (K_D^{app}) of 68 nM, a remarkably tight interaction for an inhibitor of Lyp activity. (The few known selective inhibitors of wild-type Lyp have inhibition constants in the range of 1–5 μM .^{19,20}) In a cell, the concentration of FIAsH required for potent Lyp-CCPGCC inhibition would depend on Lyp-CCPGCC's cellular concentration. Nevertheless, our *in vitro* data predict that FIAsH-induced Lyp-CCPGCC inhibition in a mammalian cell would require administration of FIAsH at low concentrations, compared to analogous experiments with known Lyp inhibitors, potentially reducing the off-target effects inherent to applications of small-molecule drugs to complex systems.

3.5. Targeting of Lyp-CCPGCC in a complex proteomic mixture

Having established that FIAsH inhibits purified Lyp-CCPGCC, we next sought to determine whether FIAsH could specifically target Lyp-CCPGCC in the context of a complex proteome, such as a cell preparation. Since a direct *in-cell* reporter of Lyp activity is not available, we took advantage of Lyp-CCPGCC-induced FIAsH fluorescence

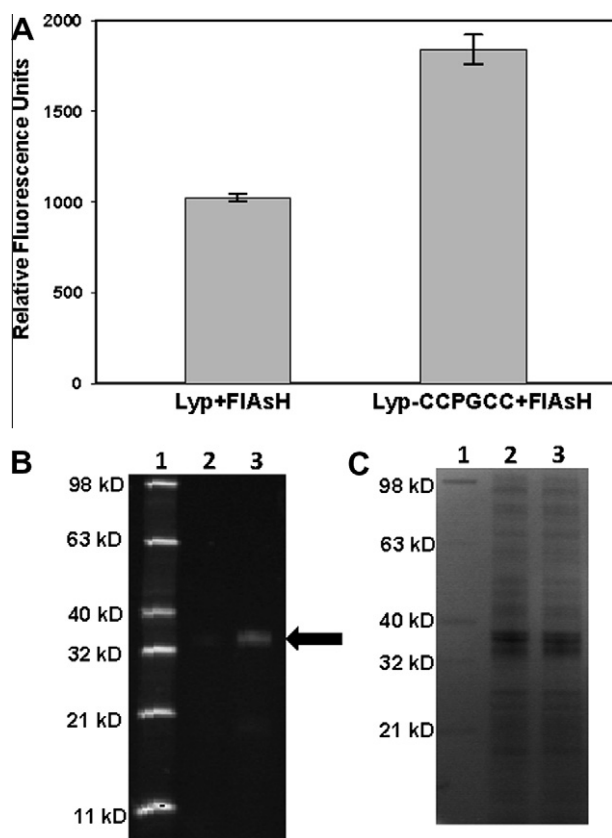


Figure 6. Targeting Lyp-CCPGCC in *E. coli* cell preparations. (A) Freeze-thawed *E. coli* cells expressing either wild-type Lyp or Lyp-CCPGCC were incubated in the presence of FIAsH (10 μ M). After 2.5 h, the FIAsH-fluorescence values of the cell suspensions were measured. (B and C) *E. coli* cells expressing either Lyp or Lyp-CCPGCC were prepared and FIAsH-treated as in (A) and subsequently lysed. Cellular proteins were separated by SDS–PAGE. FIAsH-labeled proteins were detected by fluorescence (B), followed by visualization of all proteins in the same gel by Coomassie staining (C). Lane 1: fluorescent protein standard (Invitrogen), Lane 2: lysate from Lyp-expressing cells, Lane 3: lysate from Lyp-CCPGCC-expressing cells. The black arrow indicates the prominent fluorescent 37-kD band that is enriched in Lyp-CCPGCC-expressing lysates.

as a means to potentially detect Lyp-CCPGCC/FIAsH interactions in a complex milieu. Although Lyp visualization is not the primary goal of the current work (FIAsH-mediated visualization of Lyp could be accomplished simply by placing a CCPGCC tag at Lyp's N- or C-terminus³⁷), the sensitivity of fluorescence detection could provide a powerful test of FIAsH's ability to specifically target Lyp-CCPGCC in the presence of thousands of potentially competing biomolecules.

To investigate the ability of FIAsH to bind Lyp-CCPGCC in a complex mixture, we incubated freeze-thawed wild-type Lyp-expressing and Lyp-CCPGCC-expressing *E. coli* cells in the presence of FIAsH and measured the fluorescence of the resulting cell suspensions. (Although the mammalian-cell permeability of FIAsH is well established,³⁷ we found that a freeze-thaw cycle was necessary for the timely penetration of FIAsH into *E. coli* cells.) We found that wild-type-Lyp-expressing cells give rise to a significant FIAsH fluorescence when compared to a FIAsH/buffer control solution (Fig. 6A). Although wild-type Lyp does not itself cause FIAsH to fluoresce (Fig. 5A), our observed FIAsH fluorescence in the presence of Lyp-expressing bacteria is consistent with the previous finding that *E. coli* cells endogenously express at least one protein (SlyD) that binds FIAsH and induces its fluorescence.^{40,45} We hypothesize, therefore, that the FIAsH fluorescence from Lyp-expressing bacteria results from the SlyD/FIAsH interaction, with possible low level contributions from other *E. coli* gene products.

Importantly, measured FIAsH fluorescence in the presence of Lyp-CCPGCC-expressing *E. coli* was reproducibly greater (approximately 80%) than that of wild-type-Lyp expressing cells (Fig. 6A). To further investigate the origins of the greater bulk fluorescence in the Lyp-CCPGCC-containing proteome, we separated proteins from the FIAsH-treated cell preparations by SDS–PAGE. By in-gel detection of FIAsH fluorescence, we observed a single band at 37 kD—the molecular weight of Lyp's catalytic domain—that is enriched in lysates from the Lyp-CCPGCC expressing cells (Fig. 6B). (Overexposure of the image allows for detection of other low-fluorescence bands in both lanes, including one whose observed molecular weight of 27 kD is consistent with that of SlyD, data not shown.) Since the two cell populations used in these experiments differ only by the presence of six amino acids in a single protein, the observed differences in bulk and 37-kD FIAsH fluorescence unambiguously derive from FIAsH binding to its target sequence in the Lyp-CCPGCC. These results represent the first demonstration that FIAsH can be used to target PTPs for inhibition in complex proteomic mixtures and lay the groundwork for future PTP targeting in eukaryotic cells.

4. Conclusions

Lymphoid-specific protein tyrosine phosphatase (Lyp) is an important leukocyte-signaling molecule and a putative anti-auto-immune therapeutic target. Small molecules that can specifically control cellular Lyp activity are thus important chemical-biology tools, both in basic signaling studies and in the potential drug-target validation of Lyp. We have shown that insertional mutagenesis can be used to generate a Lyp mutant (Lyp-CCPGCC) that is sensitive to inhibition by a small molecule (FIAsH) which does not inhibit Lyp or any other wild-type PTP tested to date. Lyp-CCPGCC was rationally engineered to display a FIAsH-binding peptide that does not disrupt its inherent PTP activity in the absence of added ligand. Upon addition of FIAsH, the activity of Lyp mutant is strongly inhibited, even under experimental conditions in which FIAsH and Lyp-CCPGCC are present at almost equal concentrations. We have also shown that Lyp-CCPGCC can be targeted in cell preparations of *E. coli* that express the target protein. Lyp-CCPGCC/FIAsH thus represents an 'orthogonal' PTP/inhibitor pair that can be used to control Lyp activity, potentially providing a tool for elucidating Lyp's functions in mammalian cells and engineered organisms.

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