High-throughput screen using a single-cell tyrosine phosphatase assay reveals biologically active inhibitors of tyrosine phosphatase CD45

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Edited by Arthur Weiss, University of California, San Francisco, CA, and approved July 16, 2012 (received for review March 27, 2012)

Many cellular signaling events are regulated by tyrosine phosphorylation and mediated by the opposing actions of protein tyrosine kinases and phosphatases. Protein tyrosine phosphatases are emerging as drug targets, but poor cell permeability of inhibitors has limited the development of drugs targeting these enzymes [Tautz L, et al. (2006) *Expert Opin Ther Targets* 10:157–177]. Here we developed a method to monitor tyrosine phosphatase activity at the single-cell level and applied it to the identification of cell-permeable inhibitors. The method takes advantage of the fluorogenic properties of phosphorylated coumaryl amino propionic acid (pCAP), an analog of phosphotyrosine, which can be incorporated into peptides. Once delivered into cells, pCAP peptides were dephosphorylated by protein tyrosine phosphatases, and the resulting cell fluorescence could be monitored by flow cytometry and high-content imaging. The robustness and sensitivity of the assay was validated using peptides preferentially dephosphorylated by CD45 and T-cell tyrosine phosphatase and available inhibitors of these two enzymes. The assay was applied to high-throughput screening for inhibitors of CD45, an important target for autoimmunity and infectious diseases [Hermiston ML, et al. (2003) *Annu Rev Immunol* 21:107–137]. We identified four CD45 inhibitors that showed activity in T cells and macrophages. These results indicate that our assay can be applied to primary screening for inhibitors of CD45 and of other protein tyrosine phosphatases to increase the yield of biologically active inhibitors.

single-cell assay | peptide substrate

Protein tyrosine phosphatases (PTPs) are important regulators of signal transduction and are emerging as drug targets for common and debilitating human diseases, including cancer, diabetes, arthritis, and infectious diseases (1). The transmembrane PTP CD45, a critical regulator of signaling through the T-cell receptor (TCR), was one of the first PTPs to be considered as a drug target for treatment of human autoimmune diseases (2). In addition, inhibitors of PTP-1B and SHP-2 currently are being sought for therapy of metabolic diseases and cancer, respectively (3–5). Unfortunately, development of small-molecule inhibitors of PTPs has been a challenging task. Poor cell permeability of PTP inhibitors has been a limitation difficult to overcome in later stages of drug development (5). Several methods to assess intracellular PTP activity are available (6–8), yet no assay has been able to capture intracellular PTP activity at the single-cell level. Cell-based PTP assays that work at the single-cell level and are suited to high-throughput screening might accelerate PTP inhibitor development.

In this report we describe the development and optimization of a method to monitor PTP activity at the single-cell level by using cell-permeable fluorogenic peptides that, once delivered

into cells, are dephosphorylated by PTPs. The dephosphorylation of these peptides by PTPs generates a signal that can be monitored by flow cytometry and high-content imaging. By using peptides that are preferentially dephosphorylated by selected PTPs, we tailored our assay to detect intracellular enzyme inhibition by cell-permeable compounds. The optimized assay was sensitive and robust and was applied successfully to the screening of libraries for small-molecule inhibitors of CD45, identifying four inhibitors. As expected, when incubated with human T cells, all four compounds led to increased phosphorylation of Lck on tyrosine residues 505 (Y505) and 394 (Y394), primary substrates of CD45 in T cells (2, 9). These compounds also increased signaling through the TCR, and, consistent with our previous report that CD45 inhibition is protective against *Bacillus anthracis* infection (10), they promoted macrophage viability in a cellular anthrax lethal toxin (LT) cytotoxicity assay.

Results

Recently, we reported the synthesis of a fluorogenic, phosphotyrosine-mimetic amino acid, phosphorylated coumaryl amino propionic acid (pCAP) (Fig. 1A) (11). The pCAP moiety itself has minimal fluorescence, but dephosphorylation releases CAP, which is highly fluorescent. Replacement of phosphotyrosine with pCAP in known PTP peptide substrates results in efficient fluorogenic probes of in vitro PTP activity. Peptides containing the pCAP moiety provide a sensitive, fluorogenic assay that has been useful in inhibitor screens (12) and combinatorial peptide substrate libraries (13).

Based on the in vitro utility of these substrates, we set out to optimize the substrates for intracellular use, reasoning that fluorogenic substrates for intracellular PTP activity would be useful in both identifying and validating inhibitors of PTP activity. Initially, we synthesized the peptide Ac-EDNE-(pCAP)-TARE-NH₂ (hereafter, peptide 1) based on the sequence surrounding the activating phosphotyrosine 394 in Lck, a T-cell-specific protein tyrosine kinase and a known substrate of the PTPs CD45, lymphoid tyrosine phosphatase (LYP), and Src homology region 2 domain-containing phosphatase 1 (SHP-1) (9, 14, 15). Indeed, this peptide was dephosphorylated efficiently

Author contributions: S.M.S., R.G.P., Z.-Y.Z., S.B., A.M.B., and N.B. designed research; S.M.S., R.G.P., L.M.W., D.J.W., M.D.F., S.M., S.S.D., D.R., T.K., and S.Z. performed research; S.M.S., R.G.P., Z.-Y.Z., A.M.B., and N.B. analyzed data; and S.M.S., A.M.B., and N.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1205028109/-DCSupplemental.

Fig. 1. pCAP peptides can be used to monitor intracellular PTP activity. (A) Structure of pCAP, CAP, and Et₂pCAP. (B) Images of sea urchin oocytes taken 7 min after microinjection on a fluorescence microscope. Cells were coinjected with rhodamine and pCAP peptide 1 dissolved in DMSO (Upper) or in DMSO with 10 mM sodium orthovanadate (vanadate) (Lower). (Left and Middle) Fluorescence as detected using a DAPI or rhodamine emission filter, respectively. (Right) Phase-contrast microscopy. (C) Jurkat T cells were electroporated with 100 μM pCAP peptide 1, CAP peptide 1b, or the nonhydrolyzable Et₂pCAP peptide 1c. Graph shows fluorescence of cells 30 s or 1 min after electroporation. MFI, median fluorescence intensity. (D and E) Inhibition of intracellular pCAP dephosphorylation by vanadate. (D) Jurkat cells were preincubated for 60 min with 10 mM vanadate or buffer alone. Graphs show fluorescence of cells 1 min after electroporation with 100 μM pCAP peptide 1 or CAP peptide 1b. (E) Jurkat cells were preincubated for 5 min with increasing concentrations of vanadate. Graph shows MFI of cells 1 min after electroporation with 100 μM pCAP peptide 1 (open circles). MFI of nonelectroporated cells incubated with 100 μM pCAP peptide 1 (closed black circle) is shown.

by recombinant CD45, LYP, and SHP-1 in vitro (*SI Discussion*). Notably, this peptide was not dephosphorylated by serine/threonine phosphatases, nor was the dephosphorylated peptide phosphorylated by tyrosine kinases (*SI Discussion* and Fig. S1).

To provide a proof of principle for the use of these fluorogenic peptides in cell-based assays, peptide 1 was microinjected into sea urchin oocytes. As shown in Fig. 1B, intracellular phosphatases readily dephosphorylated the peptide. However, peptide dephosphorylation was inhibited in the presence of the pan-specific PTP inhibitor sodium orthovanadate, indicating that pCAP peptides are dephosphorylated by PTPs once delivered intracellularly. Delivery of peptide 1 into human Jurkat T cells was accomplished by electroporation. Again, the peptide was dephosphorylated readily (within 1 min) by intracellular phosphatases (Fig. 1C). Electroporation of cells with the CAP-containing peptide (Ac-EDNE-(CAP)-TARE-NH₂; hereafter, peptide 1b) provided a positive control, resulting in highly fluorescent cells, whereas electroporation with a peptide containing the nonfluorescent, nonhydrolyzable Et₂pCAP moiety (Ac-EDNE-(Et₂pCAP)-TARE-NH₂; hereafter, peptide 1c) provided a minimally fluorescent negative control (Fig. 1C). In addition, pretreatment of cells with sodium orthovanadate inhibited the fluorescence of cells electroporated with peptide 1 but had no effect on the fluorescence of cells incubated with peptide 1b (Fig. 1D), and the inhibition could be detected in a dose-dependent manner (Fig. 1E). Although efficiency of transfection could not be quantified precisely for each peptide, incubation with peptides 1, 1b, and 1c induced similar cell lethality. Assuming that the three peptides have similar intracellular penetration features, these data suggest that intracellular PTPs dephosphorylate pCAP-containing peptides, producing a fluorescent signal that can be detected by both microscopy and flow cytometry. However, these initial experiments highlighted two challenges to the use of pCAP

peptides for monitoring intracellular PTP activity and inhibition: (i) the need for a more widely applicable and less invasive method for introducing peptides into cells, and (ii), the need for PTP-selective peptides rather than peptide sequences that are readily dephosphorylated by multiple PTPs.

To achieve spontaneous intracellular peptide delivery in mammalian cells, we turned to cell-penetrating peptides such as the antennapedia peptide, polyarginine, and polyarginine with a lipid tail (Fig. 2A) (16, 17). The addition of both polyarginine and a lipid tail resulted in the most efficient internalization, with myristoyl- β Ala-(Arg)₇-CAP-NH₂ (C₁₄-R₇-CAP; hereafter CAP-CPP) providing the optimal combination. Longer lipid chains did not increase uptake efficiency, as shown in Fig. 2B. Confocal microscopy of cells incubated with CAP-CPP confirmed that the peptide was internalized to the cytosol (Fig. 2B and Fig. S2).

To design probes selective for one member of the PTP family of enzymes, we took a two-pronged approach. First, because the charge and hydrophobicity of surfaces surrounding the catalytic pocket vary considerably among PTPs (15, 18–20), we explored whether the addition of the polyarginine tag with a lipid tail restricts the selectivity of the peptides. A cell-penetrating version of peptide 1 (myristoyl- β Ala-(Arg)₇-EDNE-(pCAP)-TARE-NH₂; hereafter, SP1) was synthesized. As described earlier, the SP1 peptide sequence is derived from the sequence surrounding Y394 in Lck, a known biological substrate for SHP-1, LYP, and CD45 (9, 14, 15). We then analyzed the selectivity of the probe against a panel of cytosolic PTPs in relation to the expression levels of the same PTPs in Jurkat T cells (Fig. S2). The addition of the myristoyl-polyarginine tag virtually eliminated the activity of SHP-1 [which is expressed prominently in Jurkat T cells (Fig. S2)] and of LYP on this probe in vitro. However, CD45 retained the ability to hydrolyze this peptide

Fig. 2. Single-cell assay for PTP activity using cell-permeable pCAP peptides. (A and B) Flow cytometry analysis of cell-permeable CAP uptake by Jurkat T cells. (A) Cells were incubated for 1 h with 12.5 μ M CAP-R8, C₁₄-R7-CAP (CAP-CPP), medium alone, or non-cell-permeable CAP, and analyzed by FACS. a.u., arbitrary units. (B) Cells were incubated with 10 μ M CAP-CPP, C₁₆-R7-CAP, C₁₈-R7-CAP, C₂₀-R7-CAP, non-cell-permeable CAP, or medium alone for 1 h, and analyzed by FACS. Inset shows representative image of a Jurkat cell incubated with 10 μ M CAP-CPP for 1 h. (C) The cell-permeable probe SP-1 is selective for CD45. In vitro assay to measure dephosphorylation of 0.1 mM SP-1. Graph shows mean $\pm$ SD of relative fluorescence units/s. (D and E) Detection of PTP activity and inhibition by high-content imaging. (D) Graph shows average cytoplasmic intensity of RAW 264.7 macrophages incubated for 1 h with 100 μ M SP1 or 40 μ M CAP-SP1 and increasing concentrations of vanadate. (E) Representative images of cells from D incubated with SP-1 or CAP-SP1 in the absence or presence of 100 μ M vanadate. Fluorescence of CAP and nuclei are pseudocolored blue and green, respectively. (F) Detection of PTP activity and inhibition by flow cytometry. Jurkat cells were incubated for 1 h with 100 μ M SP-1 or CAP-SP1 and increasing concentrations of vanadate or buffer alone.

efficiently, as shown in Fig. 2C. T-cell protein tyrosine phosphatase (TC-PTP), protein tyrosine phosphatase 1B (PTP1B), hematopoietic protein tyrosine phosphatase (HePTP), and vaccinia H1-related phosphatase (VHR) showed moderate to low activity on the probe (Fig. 2C). However, in Jurkat T cells the expression of these enzymes is threefold to more than 10-fold lower than the expression of CD45 (Fig. S2). Moreover TC-PTP is partly distributed into the nucleus (Fig. S2), where it would be unavailable to dephosphorylate the probe. Therefore we reasoned that CD45 likely would be a major contributor to the intracellular dephosphorylation of the SP1 probe in Jurkat T cells. In addition, we explored whether probe selectivity could be achieved by optimizing the peptide sequence based on known substrate-profiling data. We identified a potential TC-PTP-selective peptide sequence from the literature (21). We designed a cell-permeable analog of this peptide by attaching the myristoyl-polyarginine tail at the N terminus (myristoyl- β Ala-(Arg)₇-(PEG)₄-REGLN-(pCAP)-MVLAT-NH₂; hereafter, SP2). To minimize possible interference of the polyarginine tag with the PTP substrate selectivity, we incorporated a 4XPEG spacer between the tag and the peptide. As illustrated in Fig. S2, this peptide was predominantly dephosphorylated by TC-PTP, PTP1B, and CD45, much less so by LYP, HePTP, or VHR, and not at all by SHP-1.

We assessed whether peptide probes are suited to both quantitative high-content imaging with RAW 264.7 macrophages and flow cytometry with Jurkat T cells to monitor intracellular PTP activity. In these experiments a fluorescent positive control was obtained by substituting CAP for pCAP in the sequence of SP1 (CAP-SP1). CAP-SP1 was internalized into RAW 264.7 macrophages readily in a dose- and time-dependent manner (Fig. S3). RAW cells incubated with SP1 also showed a dose-dependent increase in fluorescence (Fig. S3), which was reduced dramatically by the addition of sodium orthovanadate (Fig. 2D and E). The same results were obtained in Jurkat T cells by flow cytometry, as shown in Fig. 2F and Fig. S3. To determine the sensitivity of the

assay in detecting lower PTP inhibitor activities, which are closer to those commonly used in the screening process, we performed a time-dependent study in which the dephosphorylation reaction was maintained far from the steady state. In cells incubated with SP1, an incubation time of 10 min enabled robust detection of phosphatase inhibition by 10 μ M vanadate (Fig. S3). These experiments demonstrate efficient delivery of peptide probes into mammalian cells as well as hydrolysis of pCAP-containing peptides, which can be inhibited by the addition of the pan-specific PTP inhibitor sodium orthovanadate. After probe concentration and incubation time were optimized, the assay was able to detect partial inhibition of intracellular PTPs by a nonselective PTP inhibitor.

The sensitivity and selectivity of the SP1 probe to intracellular CD45 activity in the optimized conditions were assessed by measuring the fluorescence of CD45-positive Jurkat T cells and CD45-null J45.01 T cells (22) after exposure to the peptide. The cellular fluorescence caused by peptide dephosphorylation was significantly lower in the CD45-null cells (Fig. 3A), and the assay was sensitive enough to detect a reduction in peptide dephosphorylation following a partial knockdown of CD45 expression in Jurkat T cells using a *PTPRC*-specific antisense oligonucleotide (Fig. 3B and Fig. S4). Further validation that the fluorescent signal seen upon incubation of Jurkat T cells with SP1 is caused by CD45-mediated dephosphorylation was obtained by coincubating the cells with known cell-permeable inhibitors of CD45 [NSC 95397 (10), which at 10 μ M caused almost 100% inhibition of CD45 but did not inhibit TC-PTP, PTP1B, LYP, or HePTP and caused negligible inhibition of VHR], TC-PTP [compound 8 (23), a very selective inhibitor with an IC₅₀ value of 4.3 nM on TC-PTP, eightfold greater selectivity than PTP1B, and no activity on CD45, HePTP, LYP, or VHR], or LYP [compound 4 (24), which inhibits LYP and HePTP with IC₅₀ values of 20 μ M and displays fivefold greater selectivity than CD45] (Fig. 3C and Fig. S4). The signal in the assay was sensitive to inhibition of CD45

Fig. 3. Single-cell assay to detect intracellular CD45 activity. (A and B) Single-cell assay detects reduction in CD45 expression levels. (A) Jurkat T cells or CD45-null J45.01 T cells were preincubated for 30 min with 1 mM vanadate or buffer alone. Graphs show fluorescence of cells after incubation for 10 min with 250 μ M SP1 or DMSO. (B) Jurkat cells were incubated for 48 h with 10 μ M CD45 antisense oligonucleotide (ASO) or control (Contr.) ASO. (Left) Fluorescence of cells after incubation for 10 min with 250 μ M SP1 or DMSO. (Right) Fluorescence of cells after staining with an anti-CD45 antibody or buffer alone. (C and D) Single-cell assay detects inhibition of CD45 activity but not TC-PTP. (C) Structures of the CD45-selective inhibitor NSC 95397 and the TC-PTP-selective inhibitor compound 8. (D) Jurkat cells were preincubated for 30 min with 10 nM compound 8, 10 μ M NSC 95397, or DMSO alone. (Left) Fluorescence of cells after incubation with 250 μ M SP1 for 10 min. (Right) Fluorescence of cells after incubation with 25 μ M CAP-CPP for 10 min.

but not of TC-PTP (Fig. 3D) or LYP (Fig. S4). The assay also was unaffected by knockdown of LYP using a *PTPN22*-specific antisense oligonucleotide (Fig. S4). Phospho-flow cytometry and Western blotting analyses further validated the inhibition of CD45 activity by NSC 95397 (Fig. S5). Comparative analysis using the NSC 95397 compound showed that the performance of the pCAP assay was at least equivalent, if not superior, to other commonly used assays in the field (Fig. S5). In line with the hypothesis that TC-PTP is a significant contributor to the intracellular dephosphorylation of the SP2 probe, the fluorescent signal of SP2 was sensitive to the TC-PTP inhibitor (Fig. S6). These data indicate that, if probes with partial *in vitro* selectivity are available, our optimized assay can capture the intracellular inhibition of the subset of PTPs active on the probe.

Having demonstrated the ability to monitor intracellular phosphatase activity at the single-cell level, we wanted to use this technology to identify inhibitors of CD45 using a cell-based screen. To this end, after further confirming that our assay possesses the reproducibility needed for application in high-throughput screening (Fig. S7), we screened a library of 44 chemical analogs of the CD45 inhibitor NSC 95397 (10) and 32 compounds with known biological activity selected from the MicroSource Delivery Systems Spectrum Collection. We used probe SP1 in Jurkat T cells to screen for cell-permeable inhibitors of CD45. Visual inspection of the pattern of cell fluorescence enabled easy elimination of fluorescent compounds (Fig. S7). A parallel screen using the CAP-SP1 probe was performed to exclude false-positive compounds that affected internalization of the probe (Fig. S7). However, the current assay does not weed out endosome/lysosome stabilizers, which would appear as false positives. Four compounds [L158429 (10), R164259, S349631, and purpurin] (Fig. 4) were identified as bona fide hits. The inhibition of CD45 activity by each of these four compounds was confirmed *in vitro* [IC_{50} values: 2.40 ± 0.63 μ M (L158429), 16.88 ± 1.07 μ M (R164259), 9.34 ± 2.07 μ M (S349631) and 5.97 ± 1.65 μ M

(purpurin); Fig. S8], and their intracellular activity on CD45 in human T cells was detectable by phospho-flow for phosphorylation of Lck on Y505 and Y394 (Fig. 5A–E). The selectivity of these four compounds was profiled against a panel of seven PTPs and found to be moderate (Table S1). The promiscuous activity of purpurin was not unexpected in light of previous reports suggesting it acts through an oxidative mechanism (25). However, when the compounds were tested in a T-cell-activation assay (induction of CD69 after triggering of the TCR) (Fig. 5F), in line with their Lck-Y394-inducing activity, they were able to boost TCR-dependent induction of CD69. We recently reported that inhibition of CD45 protects macrophages from *Bacillus anthracis* infection-induced cell death (10). Therefore we assessed the biological activity of the compounds in a macrophage viability assay after exposure to anthrax LT. As expected, the compounds increased resistance of the macrophages to LT-induced lysis in a dose-dependent manner (Fig. S8). The activity of the compounds in the CD69 and LT assay was somewhat proportional to their potency on CD45; however, because of the limited selectivity of these compounds, we cannot exclude the possibility that inhibition of other intracellular PTPs expressed in Jurkat T cells, RAW 264.7 macrophages, or even *Bacillus anthracis* contributed to the phenotype observed in cells treated with these compounds. In summary, through screening with our cell-based fluorogenic CD45 assay, we identified four CD45 inhibitors with biological activity in immune cells.

Discussion

Monitoring the intracellular enzymatic activity of signaling mediators at the single-cell level is increasingly applied in the drug-discovery field to screen chemical libraries (26), but current cell-based assays do not enable detection of intracellular phosphatase activity at the single-cell level. In this study we filled this gap by developing an assay that takes advantage of the properties of cell-permeable pCAP peptides, which are more tunable than other

Fig. 4. Single-cell assay for intracellular CD45 activity yields cell-permeable CD45 inhibitors. Identification of four cell-permeable CD45 inhibitors following screening of a library of NSC 95397 analogs (A) and a library of FDA-approved drugs (B). (Upper) Structures of hits. (Lower) Fluorescence of Jurkat T cells preincubated with test compound (10 μ M of NSC 95397 analogs and 50 μ M of FDA-approved drugs) or DMSO, followed by incubation with 250 μ M SP1 or 25 μ M CAP-CPP for 10 min. Green graphs show fluorescence of cells preincubated with 10 μ M NSC 95397, followed by incubation with 250 μ M SP1.

known fluorogenic PTP substrates. The assay can be applied to live and fixed cells, and the signal can be detected and measured by flow cytometry or by image-based high-content systems.

Using a peptide preferentially dephosphorylated by CD45, our assay could detect the inhibition of intracellular CD45 activity by cell-permeable compounds. Our data also suggest that pCAP-based probes preferentially targeting other phosphatases can be designed based on substrate-profiling information. To date, the mechanisms that govern PTP substrate specificity are poorly

understood. Consequently the design of substrates that are selectively dephosphorylated by one or a few PTPs is still a challenging problem. In our assay, the pCAP probe is exposed to the pool of intracellular PTPs. Therefore, the signal obtained for an inhibitor targeted to only a subset of the PTPs will be decreased by unabated dephosphorylation of the probe by other PTPs. Thus, the selectivity of the probe is a critical determinant and a limiting factor of the assay's performance. The ideal probe should exhibit infinite selectivity for a particular PTP. Although relatively few

Fig. 5. Effect of CD45 inhibitors on immune cell function. (A–D) CD45 inhibitors increase phosphorylation of Lck (Y505) in human T cells. (A–C) Jurkat cells were preincubated for 30 min with 10 μ M, 25 μ M, or 50 μ M L158429 (A), R164259 (B), or S349631 (C) and 10 μ M NSC 95397 or DMSO. (D) Jurkat cells were preincubated for 30 min with 50 μ M, 75 μ M, or 100 μ M purpurin, 10 μ M NSC 95397, or DMSO alone. Graphs show fluorescence of cells after staining with an anti-pLck (Y505) antibody. (E) CD45 inhibitors increase phosphorylation of Lck (Y394) in human T cells. Jurkat cells were preincubated for 30 min with 25 μ M L158429, 25 μ M R164259, 25 μ M S349631, 50 μ M purpurin, 10 μ M NSC 95397, or DMSO alone. Graphs show fluorescence of cells after staining with an anti-pSrc (Y418) antibody. (F) CD45 inhibitors increase human T-cell activation. Jurkat cells were preincubated for 30 min with 25 μ M L158429, 25 μ M R164259, 25 μ M S349631, 50 μ M purpurin, or DMSO alone, followed by stimulation for 4 h with 5 μ g/mL anti-CD3. Cell fluorescence was analyzed following staining with an anti-CD69 antibody.

PTP-selective peptides are known so far, substrate profiling for PTPs is gaining momentum, and a variety of approaches have been developed and applied successfully (21). To increase selectivity further, the chemistry of the tag or of the spacer could be varied. Promiscuous probes still might be useful to identify or develop scaffold inhibitors that have low selectivity but score high in cell permeability, toxicity, and other high-content variables.

We successfully applied the assay to the FACS-based screening of chemical libraries to identify cell-permeable inhibitors of CD45. Although CD45 is a relevant drug target in both the autoimmunity and infectious disease fields (2, 10), only a few cell-permeable inhibitors of CD45 are available (27). In this study we identified four inhibitors of CD45, one of which is a compound approved by the Food and Drug Administration. These compounds displayed variable and overall limited selectivity for CD45, but all were effective at inhibiting pathways downstream CD45 in immune cells. These data demonstrate that the use of our assay in a primary screening effectively enriches for cell-permeable PTP inhibitors with biological activity. However, they also highlight an important limitation: In its current version, our assay is mostly blind to the selectivity of the compounds—which, together with cell permeability, is a known pitfall of PTP inhibitor discovery. In the future we envision multiplexing the assay using probes preferentially dephosphorylated by different PTPs to identify inhibitors with desirable selectivity profiles.

The pCAP-based PTP assay is particularly suited for primary or secondary screening of chemical libraries by high-throughput flow cytometry because of the high sensitivity of the FACS approach (28). Our assay could complement phospho-flow or other cell-based assays by directly monitoring the effect of the compound on intracellular enzymatic activity. In addition, it offers a unique advantage when the substrate of the phosphatase is

unknown, and/or a suitable phospho-specific antibody is not available, and/or the known substrate can be dephosphorylated by additional phosphatases.

Materials and Methods

Peptides. The sequences of all peptides used in this study are listed in Table S2. Peptide synthesis is described in *SI Materials and Methods*.

Flow Cytometry-Based Assays. For the cell-permeable pCAP-peptide-based assays, Jurkat cells were incubated in 96-well plates at a concentration of 1×10^6 cells/100 μ L in RPMI medium containing 0.5% FBS, without phenol red. After incubation with peptide for the indicated times, cells were washed in flow cytometry buffer (HBSS without Ca^{2+} and Mg^{2+} , with 5 mM EDTA and 2.5 mg/mL BSA) with 10 mM sodium orthovanadate, fixed in 1% paraformaldehyde (wt/vol) at room temperature for 15 min, and stained with LIVE/DEAD Fixable Red or Near-IR Dead Cell Stain Kits (Invitrogen) according to the manufacturer's instructions. Cell fluorescence was monitored by flow cytometry using a BD LSR II or FACS Aria (BD Biosciences), exciting with the UV laser at 355 nm or the violet laser at 407 nm, respectively. pCAP/CAP fluorescence was detected with DAPI emission filters. Dephosphorylation of the SP1 probe also could be monitored using a BD FACSCanto II with excitation with the violet laser at 405 nm and detection with a Pacific Blue emission filter.

ACKNOWLEDGMENTS. We thank Krishna Kota and Robert C. Boltz for their help with high-content image analysis and Klaus Ley for critically reading the manuscript. This work was supported by an Inter-Disciplinary Zumberge grant at the University of Southern California (USC) (to N.B. and A.M.B.), by National Institutes of Health (NIH) Grants DK080165 (to N.B. and A.M.B.); GM079386 (to A.M.B.); CA126937, CA152194, and CA079954 (to Z.Y.Z.); and by the Department of Defense Chemical Biological Defense Program through the Defense Threat Reduction Agency Transformational Medical Technologies Program TMTI.DRUG.02.10.RD.001 (to R.G.P.) and TMTI0048_09_RD_T (to S.B.). S.M.S. was supported by the NIH Training Grant in Cellular, Biochemical, and Molecular Biology at USC.

1. Tautz L, Pellecchia M, Mustelin T (2006) Targeting the PTPome in human disease. *Expert Opin Ther Targets* 10:157–177.
2. Hermiston ML, Xu Z, Weiss A (2003) CD45: A critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol* 21:107–137.
3. Barr AJ (2010) Protein tyrosine phosphatases as drug targets: Strategies and challenges of inhibitor development. *Future Med Chem* 2:1563–1576.
4. Chan G, Kalaitzidis D, Neel BG (2008) The tyrosine phosphatase Shp2 (PTPN11) in cancer. *Cancer Metastasis Rev* 27:179–192.
5. Blaskovich MA (2009) Drug discovery and protein tyrosine phosphatases. *Curr Med Chem* 16:2095–2176.
6. Meng TC, Hsu SF, Tonks NK (2005) Development of a modified in-gel assay to identify protein tyrosine phosphatases that are oxidized and inactivated in vivo. *Methods* 35:28–36.
7. Persson C, Kappert K, Engström U, Ostman A, Sjöblom T (2005) An antibody-based method for monitoring in vivo oxidation of protein tyrosine phosphatases. *Methods* 35:37–43.
8. Karisch R, et al. (2011) Global proteomic assessment of the classical protein-tyrosine phosphatome and “Redoxome”. *Cell* 146:826–840.
9. Zikherman J, et al. (2010) CD45-Csk phosphatase-kinase titration uncouples basal and inducible T cell receptor signaling during thymic development. *Immunity* 32:342–354.
10. Panchal RG, et al. (2009) Reduced expression of CD45 protein-tyrosine phosphatase provides protection against anthrax pathogenesis. *J Biol Chem* 284:12874–12885.
11. Mitra S, Barrios AM (2005) Highly sensitive peptide-based probes for protein tyrosine phosphatase activity utilizing a fluorogenic mimic of phosphotyrosine. *Bioorg Med Chem Lett* 15:5142–5145.
12. Karver MR, Krishnamurthy D, Kulkarni RA, Bottini N, Barrios AM (2009) Identifying potent, selective protein tyrosine phosphatase inhibitors from a library of Au(I) complexes. *J Med Chem* 52:6912–6918.
13. Mitra S, Barrios AM (2008) Identifying selective protein tyrosine phosphatase substrates and inhibitors from a fluorogenic, combinatorial peptide library. *Chem-BioChem* 9:1216–1219.
14. Mustelin T, Taskén K (2003) Positive and negative regulation of T-cell activation through kinases and phosphatases. *Biochem J* 371:15–27.
15. Yu X, et al. (2007) Structure, inhibitor, and regulatory mechanism of Lyp, a lymphoid-specific tyrosine phosphatase implicated in autoimmune diseases. *Proc Natl Acad Sci USA* 104:19767–19772.
16. Patel LN, Zaro JL, Shen WC (2007) Cell penetrating peptides: Intracellular pathways and pharmaceutical perspectives. *Pharm Res* 24:1977–1992.
17. Pham W, Kircher MF, Weissleder R, Tung CH (2004) Enhancing membrane permeability by fatty acylation of oligoarginine peptides. *ChemBioChem* 5:1148–1151.
18. Barr AJ, et al. (2009) Large-scale structural analysis of the classical human protein tyrosine phosphatome. *Cell* 136:352–363.
19. Nam HJ, Poy F, Saito H, Frederick CA (2005) Structural basis for the function and regulation of the receptor protein tyrosine phosphatase CD45. *J Exp Med* 201:441–452.
20. Orrú V, et al.; Italian Collaborative Group (2009) A loss-of-function variant of PTPN22 is associated with reduced risk of systemic lupus erythematosus. *Hum Mol Genet* 18:569–579.
21. Sun H, Tan LP, Gao L, Yao SQ (2009) High-throughput screening of catalytically inactive mutants of protein tyrosine phosphatases (PTPs) in a phosphopeptide microarray. *Chem Commun (Camb)* (6):677–679.
22. Koretzky GA, Picus J, Schultz T, Weiss A (1991) Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc Natl Acad Sci USA* 88:2037–2041.
23. Zhang S, et al. (2009) Acquisition of a potent and selective TC-PTP inhibitor via a stepwise fluorophore-tagged combinatorial synthesis and screening strategy. *J Am Chem Soc* 131:13072–13079.
24. Stanford SM, et al. (2011) Discovery of a novel series of inhibitors of lymphoid tyrosine phosphatase with activity in human T cells. *J Med Chem* 54:1640–1654.
25. Gomer CJ, et al. (1996) Photodynamic therapy-mediated oxidative stress can induce expression of heat shock proteins. *Cancer Res* 56:2355–2360.
26. Barr AJ, Knapp S (2006) MAPK-specific tyrosine phosphatases: New targets for drug discovery? *Trends Pharmacol Sci* 27:525–530.
27. Lee K, Burke TR, Jr. (2003) CD45 protein-tyrosine phosphatase inhibitor development. *Curr Top Med Chem* 3:797–807.
28. Edwards BS, et al. (2009) High-content screening: Flow cytometry analysis. *Methods Mol Biol* 486:151–165.