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Rational Design of a Dephosphorylation-Resistant Reporter Enables Single-Cell Measurement of Tyrosine Kinase Activity

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Abstract

Although peptide-based reporters of protein tyrosine kinase (PTK) activity have been used to study PTK enzymology *in vitro*, the application of these reporters to intracellular conditions is compromised by their dephosphorylation, preventing PTK activity measurements. Non-proteinogenic amino acids may be utilized to rationally design selective peptidic ligands by accessing greater chemical and structural diversity than is available using the native amino acids. We describe a peptidic reporter that, upon phosphorylation by the epidermal growth factor receptor (EGFR), is resistant to dephosphorylation both *in vitro* and *in cellulo*. The reporter contains a conformationally constrained phosphorylatable moiety (7-(*S*)-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) in the place of L-tyrosine and is efficiently phosphorylated in A431 epidermoid carcinoma cells. Dephosphorylation of the reporter occurs 3 orders of magnitude more slowly compared with that of the conventional tyrosine-containing reporter.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.5b00667. Details of synthesis and assay protocols, cell culture, enzyme progress curve fitting, reporter selectivity studies, and *in vacuo* electrostatic potential maps of EGFR and PTP1B (PDF)

Notes

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Abnormal protein tyrosine kinase (PTK) activity is an established driver of oncogenesis, making these enzymes important drug targets for precision medicine.^{1–3} Patient selection for targeted PTK inhibitors has driven the development of analytical tools that can reliably measure aberrant PTK activity in clinical specimens. The inherent heterogeneity of clinical specimens necessitates that such assays be performed at the level of the single cell to permit identification of potentially drug-sensitive or -resistant subpopulations within the heterogeneous samples.^{4,5} Directed metabolic cytometry,⁶ a subtype of chemical cytometry, applies ultrasensitive capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) to quantify enzymatic modification of fluorophore-labeled peptidic substrates in single cells.⁷ These peptide substrates, or reporters, can be loaded into adherent or nonadherent cells, either cultured or primary, followed by rapid lysis of individual cells and quantification of reporter modification by CE-LIF. The separation component of this assay is particularly powerful because it enables all biochemical modifications of the reporter, and not only the reaction of interest, to be monitored. This reduces assay ambiguity and allows for more reliable measurements. Directed metabolic cytometry has been applied to single-cell measurements of protease,^{8–11} phosphatase,^{12,13} serine/threonine protein kinase,^{14,15} and lipid kinase^{16,17} activities in cultured and primary cells. However, prior to this work, no measurements of PTK activity have been achieved using this method, primarily due to the susceptibility of most PTK reporters to rapid dephosphorylation by intracellular protein tyrosine phosphatases (PTPases).

The quality of a kinase assay based on directed metabolic cytometry is largely dependent upon the characteristics of the peptide substrate used to report kinase activity. Peptide substrates of PTKs are widely employed *in vitro* to quantitatively monitor PTK biochemical activity.^{18–21} Facile synthesis, customizable chemistry, ease of handling, and compatibility with sensitive analytical instrumentation make peptidic PTK reporters powerful and broadly applicable tools for understanding PTK biochemistry. Historically, the limited selectivity of these reporters has hindered their widespread implementation *in cellulo*. In particular, PTK reporters are typically derived from phosphorylation sites within native protein substrates and are often characterized by acidic residues proximal to the tyrosyl phosphorylation site.^{22,23} This acidity renders PTK reporters especially susceptible to rapid dephosphorylation by protein tyrosine phosphatases (PTPases),^{12,24,25} resulting in significant and often overwhelming assay interference that can prevent reliable quantitation of PTK activity in intact cells. When measuring PTK activity in lysed cells, it is customary to employ a suite of

chemical inhibitors to minimize biochemical assay interference from PTPases, proteases, and off-target kinases. This tactic is not ideal for measurements in intact cells because chemical inhibition is likely to disrupt the delicate signaling architecture being studied. An inhibitor-free solution to such interference would be ideal. We report an inhibitor-free approach to mitigating PTPase and protease assay interference in an *in cellulo* PTK activity assay in which the reporter itself is rationally modified to balance the kinetics of dephosphorylation and phosphorylation, permitting the equilibrium between PTKs and PTPases to be quantified. We apply this rationally designed reporter to single-cell measurements of PTK activity in cultured cells using directed metabolic cytometry.

RESULTS AND DISCUSSION

Rational Design of PTK Reporter Using Conformational Constraint

Nonproteinogenic amino acids have been utilized to refine peptidic reporter selectivity in biological matrices by exploiting the increased structural diversity accessible through synthetic chemistry.^{26–28} Donella-Deana et al. reported that substitution of natural tyrosine with conformationally constrained, phosphorylatable analogues within existing PTK substrates produced reporters with significantly enhanced selectivity for spleen tyrosine kinase (Syk) over other cytosolic PTKs, including the Src family kinase Lyn.²⁹ The best performing of these was a highly acidic peptide containing 7-(*S*)-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (L-Htc; Figure 1B) in the place of tyrosine. We posited, based on an existing crystal structure of a phosphotyrosine peptide bound to a recombinant PTPase,³⁰ that the conformational constraint at the phosphorylation site would impede efficient dephosphorylation of the reporter. Similar to tyrosine, the L-Htc residue contains a phosphorylatable hydroxyl positioned on a rigid, conjugated six-member ring. In contrast to tyrosine, however, this ring is cyclized via a methylene bridge to the α -nitrogen, forming a fused bicyclic system. This cyclization severely restricts the flexibility of the entire residue, limiting the conformations that the peptide is able to adopt within the enzyme active site. We propose that the loss of conformational freedom in the Htc reporter would slow its dephosphorylation by PTPases and permit detection of the phosphorylated reporter *in cellulo*. As a proof of concept, we synthesized a fluorescent version of the Htc reporter (Htc-tide, 6FAM-EDDE-(L-Htc)-EEV-CONH₂) and its tyrosine analogue (Tyr-tide, 6FAM-EDDE-Y-EEV-CONH₂) and compared their kinetics of phosphorylation and dephosphorylation *in vitro*. The fluorophore enables the use of CE-LIF for detailed quantitative characterization of reporter modification and ultimately enables the assay to be adapted to single-cell analysis by directed metabolic cytometry.

We first examined the effect of the conformational constraint on the kinetics of reporter phosphorylation. We chose to study EGFR as a model PTK because its preference for highly acidic peptide substrates^{31,32} results in significant overlap both in the sequences of Htc-tide and Tyr-tide and also in the recognition motifs of many PTPases.²⁴ Additionally, EGFR is an established oncogene and drug target in many epithelium-derived cancers, where single-cell EGFR activity measurements pose an exciting analytical challenge to which directed metabolic cytometry is uniquely suited. We used a CE-LIF-based *in vitro* assay and progress curve analysis to model Htc-tide and Tyr-tide phosphorylation by the recombinant EGFR

kinase domain according to the Michaelis–Menten kinetic model (Methods and Supporting Information). We found that Htc-tide is phosphorylated by EGFR with kinetics comparable to those of Tyr-tide (Table 1), indicating that the conformational constraint at the tyrosyl residue in Htc-tide does not significantly affect its function as a reporter of EGFR PTK activity.

Kinetics of Reporter Dephosphorylation

We next tested our hypothesis that a conformational constraint at the phosphorylation site would mitigate dephosphorylation of our PTK reporter by PTPases *in vitro*. Selner et al. extensively characterized the *in vitro* selectivity of 14 classical PTPases using a peptide library approach²⁴ and demonstrated an overwhelming preference for substrates comprising acidic residues proximal to the phosphorylated tyrosine. Additionally, the authors reported a range of catalytic efficiencies across the group of PTPases studied, with some enzymes (PTP1B, TCPTP, PEST) exhibiting exceptionally rapid dephosphorylation kinetics. We chose PTP1B and TCPTP as model PTPases for our *in vitro* studies because they are widely expressed in the cell types where EGFR activity is important and because their aggressive dephosphorylation of acidic peptides poses a rigorous challenge to our efforts to offset PTPase-driven assay interference.

We generated enzymatically phosphorylated Htc-tide and Tyr-tide and measured the kinetics of dephosphorylation of both reporters by PTP1B and TCPTP *in vitro* (Table 1 and Figure 2A,B). We found, in accordance with our earlier hypothesis, that while Tyr-tide is rapidly dephosphorylated by both enzymes in a dose-dependent manner, Htc-tide dephosphorylation is minimal even at the highest enzyme concentrations tested. Measurement of the Michaelis–Menten kinetics for the four enzyme–reporter pairs reveals that the conformational constraint in Htc-tide dramatically decreases the selectivity coefficient (K_M/k_{cat}) for TCPTP from 1.9×10^4 to $4.4 \mu\text{M}^{-1} \text{s}^{-1}$, a factor of 4×10^4 . Dephosphorylation by PTP1B is similarly affected, decreasing the selectivity coefficient by a factor of 2×10^3 . Interestingly, the selectivity coefficients for the PTP1B/Htc-tide and TCPTP/Htc-tide reactions are on the same order of magnitude as that of the EGFR/Htc-tide reaction (4.4, 2.3, and $11 \mu\text{M}^{-1} \text{s}^{-1}$, respectively), indicating that *in cellulo* measurements with Htc-tide would capture the true equilibrium between PTK and PTPase activity in the cytosol.

For both PTPases studied, the k_{cat} for pTyr-tide dephosphorylation is 3 orders of magnitude higher than the corresponding value for pHtc-tide. Interestingly, the K_M values for pTyr-tide and pHtc-tide are similar for both PTPases, indicating that the differential dephosphorylation observed is primarily driven by differences in the turnover number k_{cat} rather than in the ability of the two reporters to bind to the phosphatase. Examining the *in vacuo* electrostatics of the substrate binding surfaces of published X-ray crystal structure of PTP1B³⁰ in PyMOL³³ indicates large patches of enriched positive charge near the phosphate monoester binding pocket (Supporting Information Figures 6 and 7). We posit that the overwhelming acidity of both reporters drives electrostatic interactions with the substrate binding regions of PTP1B; this electrostatic affinity is reflected in the low measured K_M values. This hypothesis is in keeping with similar reasoning by Selner et al. to explain the preference across PTPases for acidic substrates.²⁴ We speculate that the introduction of the

conformational constraint into the backbone of pHtc-tide, rather than impeding binding to the phosphatase, positions the phosphate monoester unfavorably with respect to the conserved PTPase catalytic residues, thus significantly decreasing the rate of phosphate monoester hydrolysis. The ability of EGFR to recognize Htc-tide may be a function of a more balanced surface charge distribution near the substrate binding cleft of the EGFR kinase domain³⁴ (Supporting Information), resulting in a greater number of accessible binding modes for Htc-tide and a higher probability of catalytically favorable reporter binding.

Phosphorylated Reporter Lifetime in Epithelial Tumor Lysates

We next tested whether the dramatic differences in reporter dephosphorylation observed *in vitro* were limited to our model PTPases by comparing dephosphorylation of Htc-tide and Tyr-tide in bulk lysates of a panel of cell lines. We chose cell lines overexpressing one or more of the EGFR family kinases in order to facilitate comparison to *in vitro* phosphorylation data. We first examined time-dependent dephosphorylation of the reporters in lysates of A431 cells, an immortalized line derived from an epidermoid carcinoma of the vulva and overexpressing full-length wild-type EGFR (Figure 3A). A431 cells are commonly used to study cellular EGFR activity and have previously been used to study PTP1B activity in cell lysates.^{35,36} The data in Figure 3A clearly demonstrate that, while pTyr-tide is completely dephosphorylated within 5 min, only 10% of pHtc-tide is converted to Htc-tide within 30 min. We verified that the decrease in phosphorylated reporter in both cases was due to dephosphorylation by PTPases and not to proteolysis or another competing biochemical process by pretreating the lysates with 0.1 mM sodium pervanadate, a pan-PTPase inhibitor that irreversibly oxidizes the catalytic cysteine within the PTPase active site.³⁷ Loss of both phospho reporters was essentially eliminated upon pervanadate pretreatment, confirming that this phenomenon is due to PTPase activity.

The resistance of pHtc-tide to dephosphorylation is not limited to A431 lysates. We observed similar rates of dephosphorylation of both pTyr-tide and pHtc-tide in A431, SKBR3 breast carcinoma, and NCI-N87 gastric carcinoma cell lysates (Figure 3B). Rapid pTyr-tide dephosphorylation is independent of cell type; in all cases, pTyr-tide is dephosphorylated within 5 min. The reduced sensitivity to PTPase activity exhibited by pHtc-tide is also cell type-independent, which suggests that the inability to recognize and act upon the conformationally constrained reporter extends well beyond PTP1B and TCPTP. Importantly, the relative decrease in dephosphorylation kinetics due to Htc substitution is not limited to the sequence context of Htc-tide/Tyr-tide. We synthesized consensus sequence-based reporters for the PTKs Src and ALK in both the native Tyr and Htc versions and compared their dephosphorylation in A431 lysates (Supporting Information Figure 8). The Src substrates are acidic and have a net charge of -8 when phosphorylated, while the ALK substrates contain acidic, basic, and hydrophobic residues and have a net charge of zero when phosphorylated. We used recombinant Src and ALK to phosphorylate the reporters *in vitro* and then incubated the phospho reporters in A431 lysates in the absence of PTPase inhibitors. In both cases, dephosphorylation of the Htc-containing peptide is significantly reduced compared with that of the Tyr peptide, with both Htc peptides remaining $>90\%$ phosphorylated after 60 min. This finding suggests that substitution of Tyr with Htc within

PTK reporters may be a generally applicable approach to control *in cellulo* PTK assay interference from PTPases without the need for chemical inhibition.

Increased Resistance to Proteolysis in Cell Lysates

It has been previously reported that modification of short peptides with unnatural amino acids discourages proteolysis and increases intact peptide lifetime in cell lysates and intact cells.^{38–40} We chose to investigate whether substitution of tyrosine with L-Htc would confer the additional advantage of improved stability in the face of proteolysis in cell lysates. To test this possibility, we first synthesized standards of the 6FAM-labeled fragments of Tyr-tide (Supporting Information) and verified that no fragments comigrated with intact unmodified or phosphorylated Tyr-tide or Htc-tide when separated by capillary electrophoresis (Figure 4A). Tyr-tide and Htc-tide share four fragments N-terminal to the phosphorylation site. The three Htc-containing fragments of Htc-tide were not synthesized since similarities in electrophoretic mobility between Htc-tide and Tyr-tide allow us to infer approximate migration times for the remaining Htc-containing fragments. Given the excellent separation between the intact reporters and all fragments, it is improbable that the Htc-containing fragments would comigrate with either intact reporter. Having demonstrated the ability to separate intact reporter from its metabolic products by capillary electrophoresis, we evaluated breakdown of these reporters in high-concentration, inhibitor-free A431 lysates (Figure 3B). Replacement of natural tyrosine with the constrained analogue increased the stability of the reporter almost 2-fold. Therefore, Htc-tide is resistant to both PTPase- and protease-driven assay interference compared with that for Tyr-tide.

Quantifying PTK Activity in Intact Cells

We evaluated the ability of the Htc-tide reporter to monitor PTK activity in intact A431 cells loaded by pinocytosis. Both Htc-tide (Figure 5A) and Tyr-tide (data not shown) are efficiently loaded into intact A431 by this method, as evaluated by fluorescence microscopy. Co-staining with propidium iodide (PI) to indicate nonviable cells demonstrates that successfully loaded cells remain viable at least 30 min after treatment, as evidenced by lack of observable colocalization of PI and Htc-tide emission. On the basis of the *in vitro* data, we predicted that, while both reporters should be phosphorylated in intact cells, only Htc-tide phosphorylation would be detectable given its increased resistance to PTPase interference. We loaded 10^6 serum-starved A431 with either Tyr-tide or Htc-tide immediately after a 10 min stimulation with EGF to induce EGFR pathway activation. After a 35 min incubation to allow reporter modification in the cytosol, the cells were lysed in bulk to terminate biochemical reactions. The cytosolic fraction of the lysates was analyzed by CE-LIF (Figure 5B,C). While no phosphorylation was detectable in the Tyr-tide-loaded cells, robust Htc-tide phosphorylation was readily observed (Figure 5C). Peak identities were confirmed by demonstrating comigration of cellular peaks with synthetic standards (data not shown). These data indicate that the conformationally constrained L-Htc PTK reporter is a sufficiently poor substrate for intracellular PTPases to permit quantitation of PTK activity in intact cells.

Having demonstrated the ability to detect EGF-induced PTK activity using Htc-tide, we explored the selectivity of the assay for EGFR over other PTKs. We compared

phosphorylation of pinocytically loaded Htc-tide in A431 cells that had been serum-starved overnight and either left untreated or stimulated with EGF, insulin-like growth factor (IGF), or platelet-derived growth factor (PDGF) (Supporting Information Figure 9). A low level of phosphorylation was detected in unstimulated cells and in IGF- and PDGF-stimulated cells, indicating some off-target phosphorylation of the reporter. However, a 6-fold increase in Htc-tide phosphorylation was detected in EGF-stimulated cells compared to that in unstimulated cells, whereas no statistical difference in phosphorylation was observed under IGF or PDGF stimulation.

Single-Cell Analysis

We next compared the suitability of Htc-tide versus Tyr-tide for PTK activity quantitation in intact cells at the single-cell level (Figure 6). Such measurements are clinically valuable and could be exploited to guide selection of anti-PTK therapy, which is important to treatment of PTK-dependent cancers such as subsets of colorectal and nonsmall cell lung cancer.^{41–43} Either Htc-tide or Tyr-tide was loaded by pinocytosis into serum-starved, stimulated A431 cells and incubated for 35–45 min at 37 °C. Individual loaded cells were lysed and analyzed by single-cell microelectrophoresis as described in the Methods. In keeping with previous work, both reporters undergo variable proteolysis in intact cells (Figure 6A). Consistent with population data, Htc-tide proteolysis in single intact cells is slower compared to that of Tyr-tide. This is advantageous because the longer reporter lifetime increases the range of possible assay times. The ability to allow several hours for reporter phosphorylation increases the assay sensitivity and permits analysis of samples with lower intrinsic PTK activity.

While Tyr-tide was successfully loaded into cells and underwent expected levels of proteolysis, it is not detectably phosphorylated in any of the cells analyzed (Figure 6A), in keeping with previous observations of rapid dephosphorylation of phosphotyrosine peptides in intact single cells. In contrast, Htc-tide is measurably phosphorylated in all of the cells analyzed, indicating excellent resistance to assay interference from PTPases. Quantification of Htc-tide phosphorylation in single A431 cells shows a linear increase in moles of pHtc-tide detected in response to increased total Htc-tide loaded (Figure 6B). This is consistent with estimated intracellular Htc-tide concentrations (0.001–0.1 μM) well below the *in vitro* measured K_M of 18 μM .

Conclusions

We have demonstrated that replacement of tyrosine with the conformationally constrained analogue L-Htc within a peptidic PTK reporter can be utilized to selectively control reporter dephosphorylation by PTPases while maintaining equivalent functionality as a PTK reporter. Similar rates of phosphorylation and dephosphorylation of the constrained reporter permit the equilibrium between PTK and PTPase activity to be measured *in cellulo*, whereas rapid dephosphorylation of the unconstrained reporter permits only PTPase activity to be observed. This approach to controlling reporter dephosphorylation and tuning selectivity is minimally invasive to the cell because large doses of chemical PTPase inhibitors are not required.

In this work, we have examined conformational constraint as a tool to tune the selectivity of a generic PTK reporter for PTKs over PTPases. Previous work demonstrates that conformationally constrained tyrosine analogues are recognized in varying degrees by a range of PTKs. Future efforts will explore how incorporation of conformationally constrained tyrosine analogues, in conjunction with other rational structural modifications, can be exploited to further refine the selectivity of our generic reporter for targeted measurement of EGFR PTK activity in cell lines and primary cells. Preliminary studies reveal that Htc-tide phosphorylation is reduced, but not eliminated, upon selective EGFR family PTK inhibition in A431 cell lysates (Supporting Information). Additionally, we have found that Htc-tide is phosphorylated by EGFR and Src PTKs, but not by Abl PTK, *in vitro* (data not shown). Further rational refinement of Htc-tide selectivity through optimization of the constrained tyrosine analogue at the phosphorylation site is expected to yield reporters that are selective for the target kinase over related PTKs as well as PTPases and proteases. In summary, our work suggests that conformationally constrained PTK reporters may offer a straightforward means to limit reporter dephosphorylation by off-target enzymes.

METHODS

Materials

All materials were purchased from Fisher Scientific, Sigma-Aldrich, or EMD Millipore except where otherwise indicated.

Peptide Synthesis and Purification

Peptides were prepared using Fmoc solid-phase peptide synthesis (SPPS) protocols, either by hand or using a Protein Technologies, Inc. PS3 automated peptide synthesizer. See Supporting Information for details of the synthetic protocol.

In Vitro Assays

PTK and PTPase *in vitro* assays were performed with commercially available recombinant enzymes as described in the Supporting Information. Assays were analyzed for phosphorylation or dephosphorylation as appropriate using CE-LIF as described below and in the Supporting Information.

Cell Lysate Assays

Confluent monolayers of A431 cells were rinsed with PBS, scraped over ice into lysis buffer (20 mM HEPES, pH 7.4, 5 mM β -glycerophosphate, 200 mM NaCl, 1% NP-40, 10% glycerol with 1 $\times$ Roche complete EDTA-free protease inhibitor), and incubated on ice 20 min with occasional inversion. Lysates were clarified by centrifugation at 4 °C for 15 min at 14 000 *rcf*. Supernatants were stored at –20 °C in working aliquots for up to 6 months. Details of phosphorylation, dephosphorylation, and proteolysis assays in these supernatants are available in the Supporting Information.

Capillary Electrophoresis

Capillary electrophoretic analyses of *in vitro* and lysate assay samples were performed using commercial Beckman Coulter automated PA800 CE-LIF instruments outfitted with 488 nm solid-state lasers (Coherent). Separations were carried out in 30 μm i.d. bare silica capillaries filled with electrophoretic buffer (0.2 M boric acid/sodium hydroxide, pH 9.5) under a 600 V cm^{-1} applied electric field. Prior to analysis, samples were diluted to 100–200 nM in 50% electrophoretic buffer/50% water to promote sample stacking.

Pinocytic Loading for Single-Cell EGFR Activity Assays

Monolayers of A431 cells were serum-starved overnight in DMEM medium containing 0.2% serum. Immediately before pinocytic loading with peptide reporter, the monolayers were stimulated 10 min with 200 ng mL^{-1} EGF to initiate high levels of EGFR activity. Monolayers were then disadhered using 0.05% trypsin with EDTA and suspended in complete medium. Approximately 1×10^6 cells were isolated in a 1.5 mL tube and loaded with Htc-tide or Tyr-tide by pinocytosis. The cells were incubated for 10 min with hypertonic loading solution (Influx, Life Technologies) containing 100 μM peptide reporter (20 μL per 10^6 cells), followed by pinosome lysis in hypotonic DMEM to release reporter into the cytosol and initiate the EGFR biochemical assay. Cells were washed once with complete medium and immediately plated on custom glass chambers coated with CellTak (Corning). Viability, assessed by trypan blue staining immediately after loading, was 81%. Cells were plated at a low density, resulting in approximately 30 adherent cells mm^{-2} after rinsing. Cells were allowed to adhere at 37 $^{\circ}\text{C}$ for approximately 35 min, followed by single-cell analysis.

Single-Cell Analysis with Microelectrophoresis

Single-cell microelectrophoresis was performed as previously described.^{10–14} Figures 10 and 11 of the Supporting Information display a diagram of the work flow. Also included in the Supporting Information is a step-by-step description of the process of single-cell electrophoresis. Cell chambers containing loaded A431 cells adhered to CellTak were placed on the stage of an inverted microscope. Extracellular buffer (33–37 $^{\circ}\text{C}$) was flowed over the cells for several minutes to remove weakly adhered or nonadherent cells. Cells were chosen for individual analysis based on acceptable morphology (round shape, intact membrane, lack of blebbing) and absence of other cells in the field of view. Selected cells were lysed individually by a rapid pulse from an Nd:YAG laser as previously described; then, the contents were electrokinetically loaded into the lumen of a 30 μm i.d. fused silica capillary positioned directly above the cell. The cellular contents including unmodified and modified peptide reporter were separated by capillary electrophoresis under a field strength of 200 V cm^{-1} and detected by laser-induced fluorescence. The optimal separation buffer for single-cell analysis was 0.2 M boric acid/sodium hydroxide with 2 mM sodium dodecyl sulfate (SDS), pH 9.5. The number of moles of reporter loaded into each cell was calculated based on the response from a known standard run at the beginning of each day. Data were collected in LabView⁴⁴ and analyzed in Matlab⁴⁵ using custom software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Abl	abelson kinase
ALK	anaplastic lymphoma kinase
CE-LIF	capillary electrophoresis laser-induced fluorescence
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Htc	7- <i>S</i> -hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
IGF	insulin-like growth factor
KD	kinase domain
PDGF	platelet-derived growth factor
PI	propidium iodide
PTK	protein tyrosine kinase
PTPase	protein tyrosine phosphatase
PTP1B	protein tyrosine phosphatase 1B
Src	Src tyrosine kinase
Syk	spleen tyrosine kinase
TCPTP	T-cell phosphatase

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Figure 1.
Structures of L-tyrosine (A) and L-Htc (B).

Figure 2.

In vitro dephosphorylation of pTyr-tide (solid) and pHtc-tide (patterned) in 10 min upon addition of various quantities of PTP1B (A) or TCPTP (B). $N = 3$.

Figure 3.

(A) Dephosphorylation of pTyr-tide and pHtc-tide over time in A431 lysates. $\square$, pTyr-tide + vehicle; $\bullet$, pHtc-tide + vehicle; $\triangle$, pTyr-tide + 0.1 mM pervanadate; $\blacklozenge$, pHtc-tide + 0.1 mM pervanadate. (B) Differential phosphorylated reporter dephosphorylation in HER kinase-overexpressing malignant epithelial cell lysates. Solid bars: pHtc-tide; patterned bars: pTyr-tide. $N=3$.

Figure 4.

(A) Standard CE separation of Htc-tide and Tyr-tide from their phospho forms and fragments; i–vii are the 6-FAM-labeled proteolytic fragments of Tyr-tide. (B) Reporter proteolysis in unrestricted A431 lysates. Intact Htc-tide (●) and Tyr-tide (○) remaining after incubation for various times in unrestricted A431 lysates ($N=3$).

Figure 5.

Htc-tide is phosphorylated in intact A431 cells, whereas pTyr-tide is not detected. (A) Htc-tide (green) loaded by pinocytosis into intact A431 cells. Cells are co-stained with propidium iodide (PI, red) to indicate nonviable cells. (B, C) Lysate of intact, serum-starved A431 cells loaded with Tyr-tide (Tyr; B) or Htc-tide (Htc; C) after stimulation with EGF. Internal standard indicated by (*). Only Htc-tide is phosphorylated in intact cells (pHtc).

Figure 6. Single-cell measurements of sensor phosphorylation and metabolism in intact A431 cells. (A) Comparison of Tyr-tide ($N=6$ cells) and Htc-tide ($N=11$ cells) phosphorylation (left) and proteolysis (right) in single cells analyzed by chemical cytometry. (B) Dependence of Htc-tide phosphorylation (black) and proteolysis (red) on total sensor loading in single cells ($N=11$ cells).

Table 1
In Vitro Michaelis–Menten Kinetics Describing Reporter Phosphorylation and Dephosphorylation

	Tyr-tide			Htc-tide		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1} \mu\text{M}^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1} \mu\text{M}^{-1}$)
EGFR	40.2 (± 4.9)	0.322 (± 0.016)	8.0	18.3 (± 2.2)	0.205 (± 0.006)	11
PTP1B ^a	3.1 (± 0.2)	10.8 (± 0.2)	3500	11.1 (± 0.5)	0.026 (± 0.001)	2.3
TCPTP ^a	2.8 (± 0.3)	54.1 (± 1.7)	19 000	12.2 (± 0.9)	0.054 (± 0.003)	4.4

^aThe substrate used was the phosphoform of the peptide.