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***In Silico* Design and *In Vitro* Characterization of Universal Tyrosine Kinase Peptide Substrates**

Laura J. Marholz^{1,‡}, Nicholas A. Zeringo^{2,‡}, Hua Jane Lou², Benjamin E. Turk^{2,*}, and Laurie L. Parker^{1,*}

¹Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 420 Washington Ave. SE, Minneapolis, MN 55455, United States

²Department of Pharmacology, Yale University School of Medicine, P.O. Box 208066, 333 Cedar Street, New Haven, CT 06520, United States

Abstract

The majority of the 90 human protein tyrosine kinases (PTKs) are understudied ‘orphan’ enzymes with few or no known substrates. Designing experiments aimed at assaying the catalytic activity of these PTKs has been a long-running problem. In the past, researchers have used polypeptides with a randomized 4:1 molar ratio of glutamic acid to tyrosine as general PTK substrates. However, these substrates are inefficient and perform poorly for many applications. In this work, we apply the KINATEST-ID pipeline for artificial kinase substrate discovery to design a set of candidate “universal” PTK peptide substrate sequences. We identified two unique peptide sequences from this set that had robust activity with a panel of 15 PTKs tested in an initial screen. Kinetic characterization with seven receptor and non-receptor PTKs confirmed these peptides to be efficient and general PTK substrates. The broad scope of these artificial substrates demonstrates that they should be useful as tools to probe understudied PTK activity.

Graphical Abstract

*Corresponding Authors: llparker@umn.edu, ben.turk@yale.edu.

‡Author Contributions

These authors contributed equally.

Notes

Dr. Laurie Parker owns equity in and serves on the Scientific Advisory Board for KinaSense, LLC. The University of Minnesota and Purdue University reviews and manages this relationship in accordance with their conflict of interest policies.

Supporting Information

Supporting information is available free of charge on the ACS Publications website. Tables used to derive the amino acid site preferences and scored and ranked sequence libraries are included as excel files. Peptide synthetic methods and characterization data are included as a pdf file.

Protein tyrosine kinases (PTKs) are responsible for phosphorylating specific tyrosine residues in substrate proteins, which can regulate a variety of cellular signaling pathways. This post-translational covalent modification can affect the activity, subcellular localization, or stability of proteins thereby modulating critical processes ranging from cell growth and proliferation, metabolism, differentiation, and migration.¹ Because deregulation of these processes are key events in cancer progression, PTKs have emerged as important drug targets, with many PTK inhibitors in current clinical use as cancer therapeutics.² One of the key ways to identify small molecule PTK inhibitors is through activity-based screens that assay phosphorylation of a substrate in the presence of the compound. For well-studied kinases, efficient peptide or protein substrates are known for use in these assays. However, availability of assays for the “orphan” kinome is less straightforward. Of the 90 receptor and non-receptor tyrosine kinases encoded in the human genome,³ up to 90% are relatively understudied with few high-quality substrates identified.

In the absence of an established substrate, a common approach is to use sequences containing tyrosine and various molar ratios of glutamic acid residues (polyGlu-Tyr)—either as a homogenous sequence (e.g. EEEEEEEEE) or more commonly as a heterogeneous polymeric material. These substrates are commercially available and often included in PTK assay kits, and have subsequently been used for decades in widespread applications to evaluate activity and to serve as pseudo-universal substrates in tyrosine kinase assays.^{4–8} Because PTKs often have strict substrate specificity, random mixtures of polyGlu-Tyr peptides are usually phosphorylated inefficiently, often to low stoichiometry.⁹ Due to the heterogeneous nature of the polymer, the same sample may contain components that a given kinase will phosphorylate, but may also harbor sequences that act as inhibitors of enzymatic activity. This inconsistency impedes accurate comparison of activity between preparations of substrate and across different assay conditions. Also, these heterogeneous polymers are not compatible with high-throughput assays commonly employed for inhibitor screening (e.g. LanthaScreenTM or AlphaScreenTM technologies and other TR-FRET applications), making it very challenging to develop new, efficient assays to target understudied kinases. Further, comparing relative activity of the same or different kinases from multiple preparations or assays is a poorly recognized confounding challenge in drug screening. Efficient, well-characterized, common substrates would enable assay standardization by calibrating specific activity between batches of enzyme and assay replicates. Among other benefits, having a standardized screening format would facilitate kinome-wide profiling of inhibitor specificity, which is essential for assessment of potential off-target activity.

Several *in silico* methods have been reported that attempt to predict the substrate specificity of kinases.^{10–13} Previous work in our laboratory developed the KINATEST-ID platform for kinase artificial substrate discovery.¹⁴ This pipeline uses a input dataset of established literature-curated kinase substrate and non-substrate sequences to produce a position-specific scoring matrix (PSSM). The PSSM provides a comprehensive assessment of the amino acid preferences for a given kinase at multiple positions flanking the tyrosine phosphosite. An *in silico* library of candidate biosensor sequences can be generated and evaluated for predicted kinase specificity based on PSSMs derived from multiple kinases. In the work reported in this manuscript, the KINATEST-ID workflow was used in a reversed fashion with the goal of designing a substrate that can be phosphorylated by as many PTKs as possible. A truly universal PTK substrate based on the actual substrate sequence preferences of a panel of PTKs would enable efficient activity assays to be designed, even for tyrosine kinases for which no substrates are currently known, and used in many applications from further study of orphan kinase biology to drug or tool compound discovery.

To identify a potential universal motif, the amino acid preference profiles for ABL, ARG, BTK, CSK, FES, FYN, HCK, JAK2, LCK, LYN, PYK2, SRC, SYK and YES kinases were analyzed, and two approaches were taken to discover sequences with universal kinase coverage. The first *in silico* library of potential sequences was generated incorporating amino acid residues that were universally preferred. The second was generated using both commonly preferred and neutral amino acids. We then synthesized an initial set of nine peptides predicted to have broad specificity and evaluated them in kinase assays. The screen identified two hit sequences that were phosphorylated by the entire panel of PTKs tested. Further kinetic analysis confirmed these peptides to act generally as robust PTK substrates.

The amino acid preferences at positions neighboring the tyrosine phosphorylation site for each of the kinases in the panel were assembled and used as previously described (Supplemental Tables S1–14).¹⁴ A certain amino acid residue was considered “favored” if it was found to be present at a given position at >2 standard deviations above the mean frequency of what would be expected given the “background” of potential tyrosine-centered sequences in a given substrate protein and any known interacting partners. We designated residues to be “neutral” to “slightly favored” as those exhibiting frequency values between –1 to 2 standard deviations from the mean, and “disfavored” as those observed at frequencies <–1 standard deviations from the mean. In the case of HCK and PYK2, additional data from positional scanning peptide library screens were included as previously done.¹⁴ We used this data in two types of approach, one “unbiased,” (library 1) and one “biased” (library 2) by choosing certain options from the potential amino acids represented in the motif. In the “unbiased” approach, the amino acids that were first, second, and third most favored by the kinases, as well the single most commonly observed neutral amino acid, were selected to generate the first sequence library. In the “biased” approach, the second library was generated by manual selection of amino acids that were either favored or neutral, and that were most commonly represented in the motifs across all kinases in the panel. These parallel approaches were used to provide sequences that could balance the dual needs of substrate efficiency as well as universal tolerance. A summary of the preferred and neutral

amino acids used to construct these libraries are included in the Supplementary Information (Figure S1).

The sequences in the two libraries were obtained by generating every permutation of the particular amino acids at each position. The first library generated 663,552 potential sequences, while the second library generated 2,500 potential sequences. These sequences were then subjected to the Screener tool of the KINATEST-ID pipeline to score and rank their predicted likelihood as substrates for the set of PTKs in the panel as previously described.¹⁴ This tool calculates the sum of the PSSM-derived scores for each sequence relative to each kinase in the panel. It then ranks the sequences according to this score to predict potential “selectivity”, in which a higher score means that more kinases are predicted to phosphorylate the sequence and a lower score meaning it may be more selective for a particular kinase. The usual workflow for this process would be to filter the resulting list to remove sequences that do not have predicted specificity to a kinase of interest. However, our goal was to identify sequences with the broadest possible activity, and so we ranked sequences according to highest total summed score for all screened kinases. A summary of the two scored and ranked libraries is included as Supplementary Information (Supplementary Tables S15 and S16). With these curated libraries in hand, we next sought to select a set of sequences for synthesis and empirical testing. The Screener tool removed the vast majority of the initial sequences from consideration, but still left several thousand high scoring sequences from which to choose. In the end, a total of nine different peptide sequences were chosen to form a pilot library for testing in a low-throughput kinase panel activity screen.

These peptides do not simply represent the nine highest scoring sequences that were output from Screener, but rather were selected based on a variety of factors, including considerations of additional details about known kinase preferences and particular chemical properties at certain positions (as discussed in our previous work^{14, 15}), to introduce a measure of diversity in this pilot library. The rationale for the sequence selection, synthetic procedures and characterization data for these peptides are included as Supplementary Information. The final list of peptides, their sequences and their summed score from Screener are included in Table 1.

This pilot library was then subjected to an initial screen to test their phosphorylation by a panel of 15 PTKs that included 12 non-receptor TKs (representing all 10 NRTK families) and 3 receptor TKs (representing 3 RTK families). Pilot peptides were assayed alongside several previously reported substrates that were designed to be specific to individual kinases.¹⁵ Conversion to product was monitored via incorporation of phosphate from [γ -³³P] radiolabeled adenosine triphosphate (ATP). These intensities were normalized to background and visualized as a heat map in Figure 1. Peptides **2**, **5** and **6** were phosphorylated by every PTK tested. By contrast, as previously reported, peptides that had been designed to specifically target individual kinases had more restricted reactivity. Peptides **2** and **5** had the highest average rate of phosphorylation across the entire panel of kinases (Fig. 1B), in most cases as high as or even higher than that observed for the positive control peptides for a given kinase, and were chosen for subsequent characterization.

To more fully characterize their properties as substrates for the PTKs tested, we performed follow-up kinetic analyses with seven of the kinases from the panel (non-receptor TKs BTK, SYK, ABL and SRC, and receptor TKs ALK, EGFR and FLT3). Results for each kinase-peptide combination are summarized in Table 2. In most cases K_m values were too high ($>150\ \mu\text{M}$) for individual parameters to be accurately determined, and for the remaining kinase-substrate pairs the K_m was in the double-digit μM range. As screening substrates are typically used at low μM concentrations, we used the k_{cat}/K_m value (the rate constant at low sub-saturating peptide concentration, also called the specificity constant) to compare substrates, and we extrapolated from this value a lower limit for the k_{cat} value where it could not be determined. While we found substantial variability between replicates for some kinases, relative rates of phosphorylation of the two peptides were highly consistent. For a given kinase, specificity constants for the two peptides were generally within two-fold of each other. However, some kinases had a more substantial preference for either peptide **2** (EGFR) or peptide **5** (ABL and BTK). All non-receptor TKs had k_{cat}/K_m values $>60\ \text{M}^{-1}\ \text{sec}^{-1}$ for at least one substrate.

Kinetic parameters were generally less favorable than those reported for individually optimized consensus substrates,¹⁵ which was anticipated as generating peptides with broad reactivity necessitates the presence of sub-optimal residues at some positions. Somewhat surprisingly, we found that for ABL and SRC, the catalytic parameters for peptides **2** and **5** rivaled those determined with consensus peptides. These results suggest that these two kinases are likely to be flexible in their primary sequence requirements. The lower activity for the three receptor TKs tested (FLT3, EGFR and ALK) in comparison with the NRTKs may reflect the basal activity of these kinases, each of which has low activity in the absence of cancer-associated activating mutations.^{15, 16}

In conclusion, we used a modified workflow of the KINATEST-ID pipeline to design, completely *in silico*, a set of potential universal PTK substrate sequence motifs. It should be noted that none of the sequences in libraries 1 or 2 could be predicted to be comprehensively universal substrates based on the Screener filtering tool. The capabilities of Screener are limited by the input datasets for the PSSMs, which for understudied kinases could not be accurately modeled due to the small number (e.g. 15–20) of verified substrates identified in the literature. Nevertheless, the pilot collection of nine peptides yielded two hit sequences, a far better hit rate than typically achieved with randomized peptide libraries. These two hits, peptides **2** and **5**, were first identified in an initial kinase activity screen and then further characterized with the determination of their kinetic parameters with a panel of seven PTKs representing a number of tyrosine kinase families. Both peptides were successful substrates for all kinases tested. Among the kinases we examined, peptide **2** was generally preferred over peptide **5** for RTKs, while the reverse preference was seen for NRTKs, suggesting differential utility for distinct classes of PTK. We anticipate that these universal PTK sequences will be valuable tools to assay enzymatic activity and screen for inhibitors in cases where suitable substrates have not been previously described. Furthermore, because these sequences have such broad activity, they could be used generally as quality control tools for recombinant tyrosine kinase preparations, and might enable more standardized benchmarking of sample quality and functional stability across kinases and preparations.

Protein expression and purification

The mammalian expression vector to produce the intracellular portion of human FLT3 fused to GST (GST-FLT3^{564–993}) was generated by PCR amplifying the encoding sequence and subcloning into the BamHI and NotI sites of pEBG-2T. Mammalian expression vectors to produce BTK, SRC and SYK as GST fusion proteins were previously described.¹⁵ GST-tagged kinases were expressed in HEK293T cells and purified by affinity chromatography using glutathione Sepharose 4B as described.¹⁵ His₆-EGFR^{672–998} and His₆-ALK^{1090–1416} were expressed and purified from Sf9 insect cells as described.^{16,17} Human ABL was purchased from Life Technologies.

Universal kinase substrate screen

Biotinylated peptides (10 μ M final concentration) were arrayed in a 384-well plate in 18 μ L reaction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 0.1% *v/v* Tween-20). Reactions were initiated by adding 2 μ L kinase (to 3 – 9 ng/ μ L) and [γ -³³P] ATP (to 50 μ M, 0.025 μ Ci/ μ L) in reaction buffer. Plates were sealed and incubated at 30° C for 30 min, and then 2 μ L aliquots were transferred to streptavidin membrane (Promega SAM2 biotin capture membrane) using a pin-tool. Membranes were washed as described,¹² dried and exposed to a phosphor screen. Incorporation of radiolabel was quantified by phosphor imaging using QuantityOne software (Bio-Rad). After background correction, data were normalized such that the peptide with the greatest signal was 100%. Normalized data from at least two separate experiments were averaged and converted to heatmaps using Excel (Microsoft).

Kinetic analysis of substrate peptides

For kinetic experiments, versions of the **2** and **5** peptides were synthesized in which Lys residues were substituted for the two C-terminal glycine residues. Reactions were set up as described above, with varying concentrations of peptides (1, 2.5, 5, 7.5, 15, 25, 50, 75, 100, and 150 μ M). Final kinase concentrations were as follows: 0.3 ng/ μ L for ABL, BTK, SRC and SYK; 1.5 ng/ μ L for FLT3; 2.7 ng/ μ L for ALK and EGFR. At various incubation times (10, 20 and 30 min), 2 μ L aliquots were transferred using a pin tool to P81 phosphocellulose paper (Reaction Biology), which was washed three times in 75 mM H₃PO₄, briefly washed in acetone and allowed to dry before exposing to a phosphor screen. Incorporation of radiolabel into peptides was quantified as above. After background correction, initial rates of reaction were determined by linear regression and fit to the Michaelis-Menten equation using Prism (GraphPad). For kinase-peptide pairs that had K_m values too high to accurately fit the data to the Michaelis-Menten equation, k_{cat}/K_m values were calculated from the slope of the linear portion of the Michaelis-Menten curve ([S] $\leq$ 75 μ M). Reported kinetic parameters are the average of three separate experiments except for FLT3 (two experiments).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Initial screen of the pilot library to test substrate suitability against a panel of 15 PTKs.

B) Peptides **2** and **5** had the highest phosphorylation averaged across the kinase panel (each point is an individual kinase, lines show mean and 95% confidence interval) and were therefore designated universal substrate hits. Consensus peptides reported previously by the Turk lab for the respective kinases (labeled “ACK, LYN, BMX, SYK, CSK and ABL” in both panels) were used as controls (sequences listed in the Supplementary Information).

Table 1

A list of the nine peptide sequences selected for synthesis and initial evaluation in a kinase panel activity screen. Each of these was synthesized with an C-terminal -GGK_{biotin}GG tag for affinity capture purposes.

Peptide	Sequence	Screening sum score
1	EDDEYVTPE	805.8
2	EDPIYVTLE	933.4
3	DEDIYGTPE	1035.9
4	DEPIYDTPE	1067.2
5	DEAIYATVA	695.2
6	DEPIYDTVE	1055.5
7	EDDVYDSVP	987.1
8	EDDEYISPE	676.5
9	EDDEYATPE	667.1

Michaelis-Menten kinetic analysis for top two substrates. Mean $\pm$ sem of fits across experiment for all values; ND = not determined (rates linear over concentration range); k_{cat}/K_M for data which could not be fitted was derived from the slope of the linear portion of the titration.

Table 2

	Peptide 2			Peptide 5			2/5
	K_M (μ M)	k_{cat} (sec^{-1})	k_{cat}/K_M ($\text{sec}^{-1} \text{ M}^{-1}$)	K_M (μ M)	k_{cat} (sec^{-1})	k_{cat}/K_M ($\text{sec}^{-1} \text{ M}^{-1}$)	
FLT3	41 $\pm$ 5	0.013 $\pm$ 0.004	304 $\pm$ 63	63.5 $\pm$ 0.2	0.014 $\pm$ 0.004	215 $\pm$ 54	1.43 $\pm$ 0.07
ALK	>200	>0.02	95 $\pm$ 19	>200	>0.02	79 $\pm$ 16	1.23 $\pm$ 0.02
EGFR	>200	>0.005	26 $\pm$ 5	>200	>0.001	5 $\pm$ 1	5.8 $\pm$ 0.2
Abl	>200	>4	20100 $\pm$ 2000	101 $\pm$ 19	16.5 $\pm$ 3.5	160400 $\pm$ 5700	0.13 $\pm$ 0.01
BTk	64 $\pm$ 11	0.061 $\pm$ 0.019	970 $\pm$ 240	84 $\pm$ 16	0.26 $\pm$ 0.08	3170 $\pm$ 840	0.31 $\pm$ 0.01
Syk	>200	>2x10 ⁻⁷	1080 $\pm$ 380	>200	>0.1	560 $\pm$ 120	1.8 $\pm$ 0.2
Src	>200	>40	17500 $\pm$ 3800	>200	>2	9500 $\pm$ 2800	0.52 $\pm$ 0.07