Amino-terminal sequence determinants for substrate recognition by platelet-derived growth factor receptor tyrosine kinase

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Abstract The specificity of protein kinases has been shown to be influenced by residues near the phosphoaccepting amino acid. To examine the determinants for platelet-derived growth factor receptor (PDGFR) tyrosine kinase specificity, a peptide library with three degenerate positions N-terminal to tyrosine was constructed. After reaction with PDGFR, the most abundant phosphopeptides were isolated by immunoaffinity chromatography on a column containing monoclonal anti-phosphotyrosine antibody. Further separation of bound phosphopeptides with reverse-phase HPLC led to the identification of three optimal substrates for PDGFR: Ala-Ala-Asn-Ile-Thr-Tyr-Ala-Ala-Arg-Arg-Gly, Ala-Ala-Asn-Arg-Thr-Tyr-Ala-Ala-Arg-Arg-Gly and Ala-Ala-Leu-Ile-Thr-Tyr-Ala-Ala-Arg-Arg-Gly, where underlined residues are in the degenerate positions of the peptide library. Kinetic analyses of the three individual peptides (synthesized separately) showed these peptides to be among the best reported substrates for PDGFR. Our results expand the range of amino acid residues that have been shown to serve as recognition elements for receptor tyrosine kinases.

Key words: Tyrosine kinase; Platelet-derived growth factor receptor; Peptide library; Substrate specificity

1. Introduction

Protein phosphorylation is a crucial event involved in the regulation of intracellular processes and much work has been focused on elucidating protein kinase specificity. Synthetic peptides have proved to be powerful tools in studying the specificity of various protein kinases. Synthetic peptides based on known phosphorylation sites of natural substrates have been used to study the specificity of protein kinases such as cAMP-dependent and cGMP-dependent protein kinases [1–3], the Src family of tyrosine kinases [4,5] and receptor tyrosine kinases [6,7]. Kinetic analyses have been performed for various receptor protein tyrosine kinases using synthetic peptide substrates. Epidermal growth factor receptor (EGFR) tyrosine kinase has been shown to phosphorylate substrates such as Ile-Glu-Glu-Ala-Tyr-Leu-Gly, and peptides based on phosphorylation sites of insulin β-chain [6], $p21^{ras}$, and gastrin [8]. The platelet-derived growth factor receptor (PDGFR) is less extensively characterized. Peptides that have been used as substrates for PDGFR include angiotensin II [9] and a peptide based on the phosphorylation site of $pp60^{v-src}$ [10].

More recently, degenerate peptide libraries containing randomized residues adjacent to the phosphoaccepting residue have been used to screen for optimal substrate sequences of various protein kinases [11–14]. This approach has identified optimal substrate sequences that correlate well with previously characterized phosphorylation sites for the respective protein kinases. For example, peptide library studies on cAMP-dependent protein kinase confirmed the presence of the established RRXS motif [15] in optimal substrates [11–13]. Songyang et al. [14] showed proximal acidic residues to be important determinants for both receptor and non-receptor tyrosine kinases, a finding that correlates with the observation that a number of known tyrosine phosphorylation sites have acidic residues N-terminal to tyrosine [16]. Strategies for screening peptide libraries for phosphorylated peptides have differed. Till et al. [11] employed a liquid chromatography-electrospray mass spectrometry system to identify and quantify phosphopeptide species in a peptide library with one degenerate position. Songyang et al. [13,14] used a ferric chelating column to separate phosphorylated peptides from non-phosphorylated peptides in a peptide library with eight degenerate positions. Finally, Wu et al. [12] generated peptide libraries with five or seven randomized positions according to the process described by Lam et al. [17] in which a single peptide species is synthesized on an inert bead. After phosphorylation with [γ-^{32}P]ATP, these peptide-bound beads were dispersed in agarose solution and visualized by autoradiography, and highly phosphorylated species were identified and characterized.

We describe here an approach to studying tyrosine kinase specificity using a peptide library containing three degenerate positions and immobilized monoclonal anti-phosphotyrosine antibody to screen for optimal substrates of PDGFR. Substrates identified in this manner have been confirmed for their specificity by in vitro kinetic studies.

2. Materials and methods

2.1. General

The chloromethylstyrene resin, reagents for solid-phase peptide synthesis, and *tert*-butyloxycarbonyl (Boc) derivatives of amino acids were purchased from Peninsula Laboratories or from Advanced ChemTech. Phosphocellulose P81 discs were from Whatman and [γ-^{32}P]ATP was from NEN Dupont. Centricon-30 and Microcon-10 concentrators were purchased from Amicon. Protein concentrations were measured using the Pierce Coomassie Plus Protein Assay Reagent. Monoclonal anti-phosphotyrosine antibody resin and DePro Peptide Cleavage Kits were purchased from Sigma Chemical Corp. Peptide library synthesis was performed on a RaMPS apparatus from Dupont, and peptide purifications were performed on an ISCO 2350 High-Pressure Liquid Chromatography (HPLC) system using Vydac C18 semipreparative and analytical columns. Amino acid analyses were carried out on a Waters Picotag Amino Acid Analysis System after hydrolysis of peptide in 6 N HCl at 110°C for 24 h. Protein sequencing was performed using a Millipore Sequelon-AA Reagent

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Kit and an Applied Biosystems model 475A Pulsed Liquid Protein Sequencer. Radioactivity was measured in a Beckman LS3801 liquid scintillation counter.

Residues Lys-526–Leu-1067 of the cytoplasmic domain of mouse β-PDGFR [18] (cDNA kindly provided by Dr. L.T. Williams, University of California, San Francisco) were fused to an N-terminal FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) [19] and expressed in Sf9 cells using a baculovirus system [20]. Purification of PDGFR was performed on IBI anti-FLAG affinity gel resin, which consists of murine IgG monoclonal anti-FLAG antibody coupled to agarose.

2.2. Peptide synthesis

Individual peptides were prepared by solid-phase synthesis on Merrifield resin according to standard t-Boc protocols [21] as previously described [11]. For the peptide library, three degenerate positions were prepared on a RaMPS apparatus according to the 'divide-couple-recombine' strategy [22]. At each degenerate position, 0.4 mmol of peptide-resin was divided into 20 parts by weight and each part underwent double coupling with a 6-fold excess of activated hydroxybenzotriazole (HOBt) ester of a unique amino acid. Qualitative ninhydrin tests [23] after every individual coupling were negative. Upon completion of coupling all parts were recombined before proceeding to the next cycle. The completed peptide library was deprotected and cleaved from the resin by treatment with HF, extracted with 10% acetic acid and lyophilized. The individual peptides PR1, PR2, PR3, Control 1, and Control 2 were deprotected and cleaved from resin using DePro Peptide Cleavage Kits from Sigma according to the manufacturer's specifications. Final purification of all peptides was accomplished by reverse-phase HPLC on a Vydac C18 semipreparative column that was equilibrated in 0.1% trifluoroacetic acid. Peptides were eluted with a gradient of 0–95% solvent B (75% acetonitrile in 0.1% trifluoroacetic acid) and absorbance was measured at 220 nm. Amino acid analyses and matrix-assisted laser desorption mass spectra were consistent with all expected peptide primary structures.

2.3. Purification of PDGFR

The cytoplasmic domain of PDGFR was purified according to the protocol supplied with the IBI Anti-FLAG M2 resin with the following modifications. Anti-FLAG resin was washed once with HNA buffer (50 mM HEPES, 150 mM NaCl, 0.02% NaN_3, pH 7.4), twice with 0.1 M glycine-HCl (pH 3.0) followed by two more washes of HNA buffer and two washes of binding buffer (25 mM HEPES, 5 mM EDTA, 50 mM NaCl, 0.2% NP-40, 0.2 μM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, pH 7.4). A 1:1 mixture of PDGFR lysate and binding buffer was added to the resin and allowed to rock for 3 h at 4°C. This was followed by five washes with binding buffer, packing of the resin on a 6×1 cm column, and two washes with elution buffer (25 mM HEPES, 150 mM NaCl, 10% glycerol, 0.2 μM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mg/ml FLAG epitope peptide, pH 7.4). Fractions were collected and assayed for tyrosine kinase activity; active fractions were pooled and concentrated on a Centricon-30 concentrator. Protein concentrations were measured using the Pierce Protein Assay Reagent and concentrated enzyme was stored in 40% glycerol at −20°C.

2.4. Phosphorylation of peptides

Phosphorylation assays were carried out according to a modified version of the phosphocellulose paper assay protocol previously described [24]. Reactions were performed in 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 6 mM $MnCl_2$, 1 mg/ml bovine serum albumin, and 200 μM [γ-^{32}P]ATP, with varying peptide concentrations.

To study the kinetics of phosphorylation of individual peptides, PDGFR was allowed to autophosphorylate with non-radioactive ATP for 60 min at 30°C and reactions were initiated upon addition of peptide and [γ-^{32}P]ATP. Reactions were carried out in 25 μl volumes with varying concentrations of substrate, and were incubated at 30°C. Rates of phosphate transfer to synthetic peptides were measured as previously described [24]. Basal levels of enzyme autophosphorylation were subtracted from total phosphorylation levels to obtain peptide phosphorylation levels.

2.5. Isolation and identification of tyrosine-phosphorylated peptides

4 mM peptide library (total peptide concentration) was allowed to react with PDGFR under the conditions described above in a volume of 1 ml for 1 h at 30°C. Peptides were separated from BSA and protein kinase by filtration through a Microcon-10 unit (Amicon). The mixture of phosphorylated and unphosphorylated peptides was rocked together with anti-phosphotyrosine resin for 2 h at room temperature. The resin was then packed on a 4×0.5 cm column and unbound peptides were removed with ten 2-ml washes of phosphate-buffered saline (pH 7.0). Bound peptides were eluted with three 2-ml washes of 0.1 M glycine-HCl (pH 3.5). Fractions were analyzed by liquid scintillation counting, and glycine-HCl eluted fractions containing ^{32}P were applied to a reverse-phase HPLC column. Peaks of absorbance from HPLC were collected and analyzed by liquid scintillation counting to verify the presence of radioactivity. The sequences of the phosphopeptides were determined by Edman degradation. Peptides were linked via their C-termini to Sequelon-AA membrane using the Millipore Sequelon-AA Reagent Kit according to the manufacturer's specifications. Controls to ensure the specificity of the anti-phosphotyrosine resin were performed in an analogous manner using unphosphorylated and phosphorylated [Leu^8]src-related peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Leu-Tyr-Ala-Ala-Arg-Gly) [11].

3. Results

3.1. Synthesis of peptide library

The peptide library used in this study has the following sequence: Ala-Ala-X-X-X-Tyr-Ala-Ala-Arg-Arg-Gly, where X represents an equimolar mixture of all 20 naturally occurring amino acids. The library was designed with randomized residues N-terminal to tyrosine because substitutions at these positions in synthetic peptides have been demonstrated to influence phosphorylation by receptor and non-receptor tyrosine kinases [11,25,26]. Alanine was chosen as the amino acid to flank the randomized positions and tyrosine because it has neutral character, lacks hydrogen bonding potential, and is relatively flexible. Two arginine residues were included near the peptide C-terminus to facilitate binding to phosphocellulose paper for protein kinase assays [27]. The peptide library was synthesized by standard solid-phase synthesis [21], except at the randomized positions. For the degenerate positions, the resin was divided into 20 portions and a separate coupling reaction was carried out for each amino acid. The desired stoichiometry at the randomized positions was verified by amino acid analysis (data not shown).

3.2. Phosphorylation of peptide library

As an initial test of the reactivity of the peptide library, 1 mM peptide library (total peptide concentration) was incubated with PDGFR in the presence of [γ-^{32}P]ATP. The progress of the reaction was followed by the phosphocellulose paper binding assay. These results indicated that maximal phosphorylation of components of the library occurred after 60 min of reaction, and that approx. 0.15% of the total peptide library was phosphorylated (data not shown). The reaction between the peptide library and PDGFR was analyzed by reverse-phase HPLC and scintillation counting (Fig. 1). These data indicated that the major phosphopeptide (or phospho-

Table 1
Sequencing of phosphopeptide peaks b1 and b2

Peak b1		Peak b2	
Cycle	Amino acid	Cycle	Amino acid
3	Asn	3	Leu
4	Arg, Ile[a]	4	Ile
5	Thr	5	Thr

[a]A lower amount of Ile was detected with the major amino acid Arg (24% of Arg).

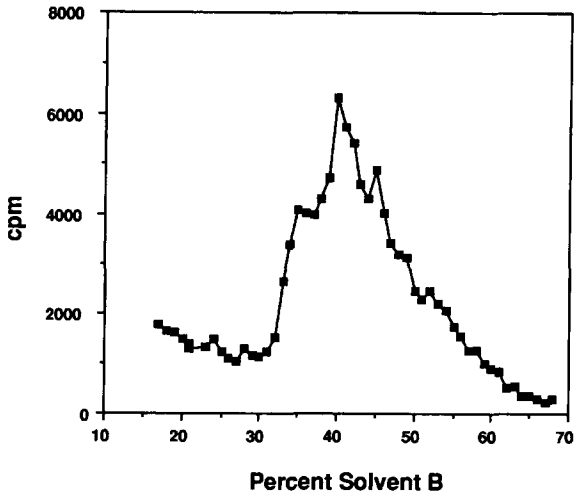

Fig. 1. HPLC analysis of PDGFR reaction with peptide library. HPLC conditions are described in Section 2. Fractions were collected every 30 s and analyzed by scintillation counting.

peptides) eluted from the column at approx. 39.5% solvent B (Fig. 1).

A library reaction with PDGFR was carried out on a larger scale to identify peptides phosphorylated by the enzyme. After 60 min of reaction, PDGFR was removed from peptides by filtration using a Microcon-10 unit (molecular mass cut-off = 10 kDa). Phosphorylated peptides in the Microcon-10 filtrate were separated from unphosphorylated peptides by immunoaffinity chromatography using anti-phosphotyrosine antibody resin.

To test the ability of the anti-phosphotyrosine column to bind phosphopeptides under the conditions used for our assay, controls were first carried out using phosphorylated and unphosphorylated [Leu^8]src-related peptide [11]. The phosphorylated version of this peptide was produced by reaction with [γ-^{32}P]ATP and the v-Abl tyrosine kinase, as previously described [11]. Unphosphorylated [Leu^8]src-related peptide did not bind to antiphosphotyrosine resin under the conditions of our assay, as determined by HPLC analysis of the fractions eluted by 0.1 M glycine-HCl, pH 3.5 (Fig. 2A). In contrast, phosphorylated [Leu^8]src-related peptide was retained on the column under the same conditions. The radioactive phosphopeptide was eluted from the immunoaffinity column by treatment with glycine-HCl and eluted at 64% B on reverse-phase HPLC (data not shown).

This procedure for binding, washing, and elution was then carried out on the products of the peptide library reaction with PDGFR. Radioactive fractions eluted from the column were pooled and analyzed by reverse-phase HPLC (Fig. 2B). Two major peaks of absorbance at 220 nm, b1 and b2, eluted at 39% and 41% B, respectively. Fractions corresponding to these peaks were collected for further characterization. Peptides from peaks b1 and b2 were linked via their C-termini onto Sequelon-AA membranes and were subjected to 7 cycles of Edman sequencing. The most abundant amino acids from relevant sequencing cycles are shown (Table 1). Cycles 3–5 correspond to the degenerate positions. Peak b1 has Asn in the −3 position, Arg in the −2 position, and Thr in the −1 position. A small amount of Ile was also observed at the −2 position (24% of that observed for Arg), suggesting that this HPLC peak contained two related phosphopeptides. Peak b2 has Leu in the −3 position, Ile in the −2 position, and Thr in the −1 position.

3.3. Kinetic analyses

Individual peptides corresponding to the sequenced phosphopeptides were synthesized to confirm the library results. Peptides PR1, PR2 and PR3 correspond to the peptide sequences of peak b1, peak b2, and the minor sequence obtained from peak b1, respectively. For comparison, a peptide with three Glu residues N-terminal to tyrosine (Ala-Ala-Glu-Glu-Glu-Tyr-Ala-Ala-Arg-Arg-Gly) was tested as a substrate for PDGFR, because peptides with multiple acidic residues have previously been shown to be substrates for receptor tyrosine kinases [8,28]. Another peptide containing alanine residues N-terminal to tyrosine (Ala-Ala-Ala-Ala-Ala-Tyr-Ala-Ala-Arg-Arg-Gly) was synthesized to test residues that are not predicted to serve as recognition elements for PDGFR. Kinetic analyses with these five peptides were performed using the phosphocellulose paper binding assay [27]. Experiments were carried out at saturating concentrations of ATP and the divalent cations Mg^{2+} and Mn^{2+} to arrive at values of K_m, V_{max} and k_{cat} for the peptides. All five peptides tested served as substrates for the cytoplasmic domain of PDGFR (Table 2). The kinetic parameters for the three peptides selected from the library compare favorably with those of the best known peptide substrates for PDGFR (e.g. src-related peptide with $K_m = 2$ mM, $V_{max} = 0.004$ nmol/min per mg [10]). Of the five peptides, PR3 is the best substrate for PDGFR, with $K_m = 58.9$ μM and $k_{cat}/K_m = 977.5$ M^{-1} min^{-1}. Peptides PR2 and PR3, both of which contained Ile at the −2 position and Thr at the −1 position, had the lowest K_m values of the peptides tested (Table 2).

4. Discussion

The use of peptide libraries provides a method to screen rapidly for optimal substrates of protein tyrosine kinases. A

Table 2
Kinetic analyses of platelet-derived growth factor receptor kinase

Peptide	Sequence	K_m (μM)	V_{max} (nmol/min per mg)	k_{cat}/K_m (M^{-1} min^{-1})
PR1	AANRTYAARRG[a]	189.4	0.37	120.0
PR2	AALITYAARRG[a]	53.9	0.59	674.7
PR3	AANITYAARRG[a]	58.9	0.94	977.5
Control 1	AAAAAYAARRG	946.5	0.07	4.7
Control 2	AAEEEYAARRG	719.5	0.10	8.5

Amino acids are written in single-letter codes.
[a]The underlined amino acids correspond to the degenerate positions in the peptide library.

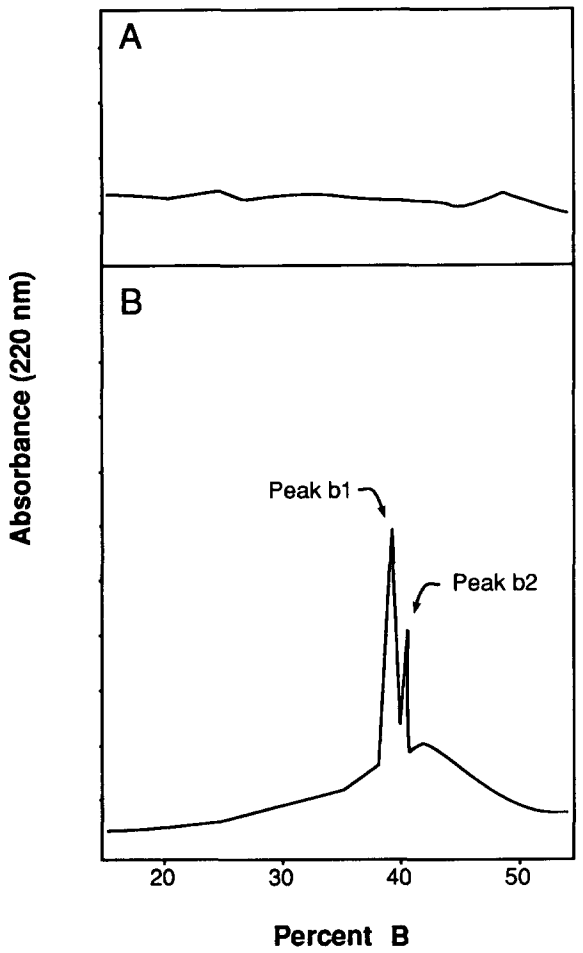

Fig. 2. HPLC analysis of peptides eluted from anti-phosphotyrosine column. Conditions are described in Section 2. (A) Control reaction with unphosphorylated [Leu^8]src-related peptide. 375 nmol of [Leu^8]src-related peptide was incubated with anti-phosphotyrosine resin, washed, and eluted as described in the text. The eluate was applied to an analytical C18 column. (B) Analysis of the products of the peptide library reaction with PDGFR. After elution from the anti-phosphotyrosine column, phosphopeptides were analyzed under identical conditions to (A).

primary advantage of this approach is the ease with which diversity may be generated in the substrate pool. Our peptide library with three degenerate positions provides a pool of 8000 different substrates; each additional degenerate position will increase that diversity by a factor of 20. Because monoclonal antibodies against phosphotyrosine are highly specific for peptides and proteins containing phosphorylated (vs. unphosphorylated) tyrosine, background levels of unphosphorylated peptide are undetectable in our screening approach [29]. In addition, these antibodies have been demonstrated to be relatively insensitive to the sequence of amino acids surrounding phosphotyrosine [30]. In our experiments, anti-phosphotyrosine immunoaffinity chromatography appears to select the major phosphopeptides from the library reaction with PDGFR, as judged by a comparison of Fig. 1 with Fig. 2B. The pool of phosphopeptides isolated by immunoaffinity chromatography was analyzed further by reverse-phase HPLC. The most abundant phosphopeptides were separated from each other prior to sequencing.

Our kinetic studies confirm the effectiveness of this library approach. Peptides PR1–PR3 were synthesized according to the sequences of the most abundant phosphopeptide species (Table 1). Peptides PR2 and PR3 were especially good substrates, among the best reported for PDGFR (Table 2) [9,10]. Kinetic analyses of peptides PR2 and PR3 compare the specificity contribution of the two determinants Leu and Asn at the −3 position. The K_m values for the peptides are similar; the V_{max} and k_{cat}/K_m values, however, are somewhat higher for peptide PR3 (Table 2). In the −2 position, Arg and Ile are the two predominant determinants (Table 1), and a comparison between peptides PR1 and PR3 indicates that Ile is preferred approx. 8-fold over Arg according to k_{cat}/K_m values (Table 2). Thr appeared to be the primary determinant in the −1 position (Table 1).

Songyang et al. [14] previously studied PDGFR kinase specificity using a peptide library with four randomized positions on each side of the acceptor tyrosine. In this case, the entire mixture of phosphorylated peptides was sequenced to elucidate the N- and C-terminal consensus sequences. Their results indicated that the preferred residues at the −4, −3, −2, and −1 positions were all Glu, and for the +1, +2, +3, and +4 positions Val, Phe, Ile, and X, respectively, where X represents any one of the 20 naturally occurring amino acids other than Trp, Cys, Tyr, Ser, or Thr. The observed difference of determinants in the N-terminal positions may be a result of the different peptide libraries used. Our library included all 20 naturally occurring amino acids in the degenerate positions and lacked degeneracy for positions C-terminal to the phosphoacceptor tyrosine. The composition of amino acids C-terminal to tyrosine may influence preferred determinants at N-terminal positions. Whether C-terminal residues and N-terminal residues interact separately with catalytic site binding pockets, or coordinate to form a single binding unit in the interaction with the active site of PDGFR tyrosine kinase, is unknown. Although the peptide substrates we used are relatively small, the contribution of substrate secondary structure, if any, to specificity cannot be ruled out. We included in our kinetics study a peptide containing three N-terminal acidic residues (Control 2) to compare to the peptides with N-terminal aliphatic hydroxy and hydrophobic side chain residues. This peptide was a poorer substrate for PDGFR than peptides PR1, PR2, and PR3, both in terms of K_m and V_{max} (Table 2). This suggests that, at least in the context of this peptide sequence, acidic residues at the -1, -2, and -3 positions are not the primary determinants of substrate specificity. A sequence alignment of mouse PDGFR with the crystal structure of apo human insulin receptor kinase [31] suggests that residues Val-690, Arg-830, and Thr-901 of PDGFR may interact with the −1 position of the bound substrate, residue His-694 with the −2 position, and residue Lys-697 with the −3 position (P. Chan and W.T. Miller, unpublished). Structural studies of kinase-substrate complexes are needed to determine whether the N-terminal residues with acidic side chains interact with the peptide binding site of PDGFR in a manner different than those with hydrophobic and aliphatic hydroxy side chains.

Further diversity in the substrate pool can be easily generated by incorporating more degenerate positions in the peptide library, and the contribution of residues to C-terminal to tyrosine to kinase specificity can be explored by adding degeneracy at these sites. Non-naturally occurring amino acids or D-amino acids can be added to increase diversity beyond the

limits set by the 20 natural amino acids and to explore with greater resolution the chemical nature of specificity. This approach is relevant to laying the foundation for rational design of protein kinase inhibitors. In addition, optimal substrate motifs may be used to search available databases for possible natural substrates of protein tyrosine kinases.

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