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CD45 PROTEIN TYROSINE PHOSPHATASE: DETERMINATION OF MINIMAL PEPTIDE LENGTH FOR SUBSTRATE RECOGNITION AND SYNTHESIS OF SOME TYROSINE-BASED ELECTROPHILES AS POTENTIAL ACTIVE-SITE DIRECTED IRREVERSIBLE INHIBITORS

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Abstract: Using *fyn* PTK as a template, a series of phosphopeptides **1-11** spanning in length from 1-14 amino acids was prepared. Kinetic evaluation of **1-11** suggest that CD45 does not have a strong preference for its N- or C-terminal amino acids and that extended phosphopeptides are not required for efficient substrate turnover.

CD45 is a protein tyrosine phosphatase expressed on the surface of T-cells and other nucleated hematopoietic cells.[1] The enzyme catalyzes the hydrolysis of the O-P bond in O-phosphorylated tyrosine residues of certain protein tyrosine kinases (PTKs).[1b] Dephosphorylation of these tyrosine kinases by CD45 results in the up-regulation of their catalytic activity, initiating a cascade of intracellular events leading to T-cell activation.[2] CD45 therefore represents a therapeutic target for certain autoimmune and chronic inflammatory diseases characterized by aberrant T-cell activation.

CD45 interacts with members of the src-family PTKs, most notably *c-fyn* and the T-cell specific kinase *lck*.[1b,3] The secondary amino acid sequence of the src PTKs surrounding the site of tyrosine dephosphorylation reveals a high degree of sequence homology. (Figure 1).[3] Using *fyn* PTK as a template, a series of N-acetyl phosphopeptide amides **1-11** spanning in length from 1-14 amino acids was synthesized and evaluated as substrates for CD45. Our intent was to ascertain the smallest phosphopeptide sequence required by the enzyme for substrate recognition and to use this information as a starting point for the rational design of peptide-based inhibitors.

Figure 1. C-Terminal Sequences for the *src* Family PTKs.

src	FTSTEPQ**Y**QPGENL
fyn	FTATEPQ**Y**QPGENL
lck	FTATEGQ**Y**QPQP--
lyn	YTATEGQ**Y**QQQP--
yes	FTATEPQ**Y**QPGENL
hck	FTSAEPQ**Y**QPGDQT
fgr	YTATESQ**Y**QQQP--
blk	YTATEGQ**Y**ELQP--

Phosphopeptides **1-11** were prepared using standard solid phase peptide synthetic protocols.[4] The k_{cat}, K_m and k_{cat}/K_m kinetic constants for each of the phosphopeptides **1-11** were determined using purified CD45 and the malachite green assay (Table 1).[5] The k_{cat}/K_m specificity constant for peptide **1**, the longest peptide (14 amino acids) generated in this study, was found to be 8.1 x 10^{-3} $M^{-1}s^{-1}$. Systematic deletion of the amino acids from the C-terminal and N-terminal ends of peptide **1** afforded the truncated phosphopeptides **2-4** and **5-7**, respectively. These truncated phosphopeptides displayed k_{cat}/K_m values ranging from *ca.* 3.5-6.5 x 10^{-3} $M^{-1}s^{-1}$, which were within 2-3 fold of the specificity constant obtained for **1**. Further truncation of peptide **1** by simultaneous removal of both the C- and N-terminal amino acids gave rise to the peptides **8-10**. Comparison of the k_{cat}/K_m values for each of these peptides to **1**, again showed that they do not differ significantly from **1**. Individual k_{cat} and K_m kinetic constants for **1-10** vary by less than a factor of 2.

It is apparent that the enzyme does not have a strong preference for either the N- or C-terminal peptide segments of its substrates.[6] The truncated N-terminal phosphopeptides **5-7** have specificity constants within two-fold of the C-terminal truncated peptides **2-4**. This is in contrast to the recent report by Dixon, where a yeast protein tyrosine phosphotase appears to have a preference, albeit only 4x, for its N-terminal amino acids.[7] At the outset of our study, we had anticipated that Gln-pTyr-Gln (QpYQ) **10** may have been the shortest phosphopeptide required for efficient substrate turnover given the fact that nearly all of the *src* family PTKs contain this tripeptide unit. However, we observed that even the N-acetyl phosphotyrosine carboxamide **11** demonstrated reasonable substrate activity in comparison to the longer substrates. Some specificity is derived from the tyrosine amino acid as *p*-nitrophenylphosphate (pNPP) **12** possesses a k_{cat}/K_m = 43 x 10^{-3} $M^{-1}s^{-1}$ versus 1.5 x 10^{-3} $M^{-1}s^{-1}$ for **11**. This difference in specificity constants between **11** and **12** is principally a reflection of the large (>10x) difference in K_m values.[8]

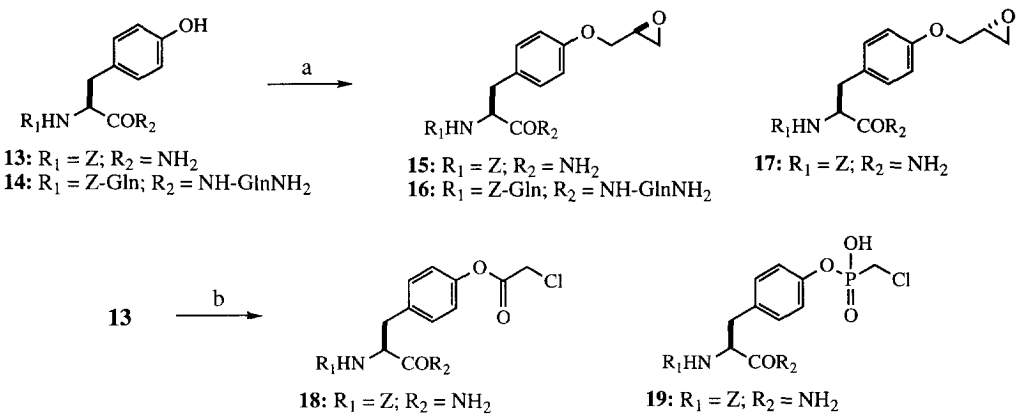
With the observation that **10** and **11** are substrates for CD45, a series of electrophilic tyrosine analogs **15-19** were prepared (Scheme 1) and examined for their ability to inhibit CD45. These agents were designed to act as irreversible inhibitors via alkylation of the active-site cysteine.[9a] When assayed against the enzyme at 200 μM, compounds **13-19** were not inhibitory (data not shown). Time-dependent inactivation of enzyme was not seen for these agents using long incubation times. Although certain benzyloxyglycidyls have been shown to irreversibly inactivate thiol phosphatases,[9a] this appears not the case for tyrosine epoxides **13-17** against CD45.[9b] Even the chloromethylphosphonate **19** which is structurally most closely related to phosphotyrosine **11** lacks affinity for enzyme.

In summary, eleven phosphopeptides based on *fyn* PTK were prepared and evaluated as substrates for CD45. The results of the kinetic study revealed that CD45 does not require extended phosphopeptides for efficient substrate turnover. The k_{cat}/K_m values for each substrate **1-11** ranged from *ca.* 3.5-8.0 x 10^{-3} $M^{-1}s^{-1}$ while individual k_{cat} and K_m constants fell within 2-fold of one another. The enzyme does however, derive some specificity from the

TABLE 1. Kinetic constants for CD45 peptide substrates

Peptide No.	Phosphopeptide	Peptide length (no. of amino acids)	k_{cat} (s^{-1})	K_m (μM)	10^{-4} X k_{cat}/K_m ($M^{-1}s^{-1}$)
1	Ac-FTATEPQ**pY**QPGQNL-NH_2	14	3.95 ± 0.0	278 ± 0.0	1.42 ± 0.0
2	Ac-FTATEPQ**pY**QPGQN-NH_2	13	4.68 ± 0.0	253 ± 0.0	1.84 ± 0.0
3	Ac-FTATEPQ**pY**QPG-NH_2	11	4.47 ± 0.0	220 ± 0.0	2.13 ± 0.0
4	Ac-FTATEPQ**pY**Q-NH_2	9	4.85 ± 0.0	261 ± 0.0	1.85 ± 0.0
5	Ac-ATEPQ**pY**QPGQNL-NH_2	12	4.93 ± 0.0	143 ± 0.0	3.45 ± 0.0
6	Ac-EPQ**pY**QPGQNL-NH_2	10	4.22 ± 0.0	102 ± 0.0	4.13 ± 0.0
7	Ac-Q**pY**QPGQNL-NH_2	8	4.82 ± 0.0	172 ± 0.0	2.81 ± 0.0
8	Ac-PQ**pY**QP-NH_2	5	3.95 ± 0.0	278 ± 0.0	1.42 ± 0.0
9	Ac-Q**pY**QP-NH_2	4	3.95 ± 0.0	278 ± 0.0	1.42 ± 0.0
10	Ac-PQ**pY**Q-NH_2	3	3.95 ± 0.0	278 ± 0.0	1.42 ± 0.0
11	Ac-**pY**-NH_2	1	3.95 ± 0.0	278 ± 0.0	1.42 ± 0.0
12	*p*-nitrophenylphosphate	0	3.95 ± 0.0	278 ± 0.0	1.42 ± 0.0

Scheme 1. Synthesis of Electrophilic Tyrosine Analogs **15-19**.



Reagents and conditions: (a) **13** or **14** (1.0 equiv), (S)- or (R)-glycidyl tosylate, 2-*t*-butylimino-2-diethylamino-1,3-dimehtylperhydro 1,2,3-diazaphosphorin (Sproat, B. S.; Beijer, B. *Nucleic Acids Rsh.* **1990**, *18*, 41), MeCN, 25 °C, 18 h, 75%; (b) $ClCH_2COCl$, DMAP, CH_2Cl_2-MeCN (2:1), 0 °C to 25 °C, 12 h, 98% or $ClCH_2P(O)(Cl)_2$, pyr., 0 °C to 25 °C, 12 h, 94%.

tyrosine residue as pNPP **12** was clearly an inferior substrate as compared to **1-11**. Our study serves to further confirm that CD45 is sequence non-specific,[8] making the prospect of identifying a substrate sequence-based specific inhibitor of CD45 most unlikely. We have further shown that CD45 is not inhibited by tyrosyl epoxides (e.g. **15-17**), -chloromethyl esters (e.g. **18**), or -chloromethylphosphates (e.g. **19**). CD45 specificity may arise through the action of co-capping of external domains in either the substrates or CD45 itself.[8]

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4. Phosphopeptides **1-11** were prepared using standard solid phase peptide chemistry protocols, employing either SASRIN (to yield C-terminal acids) or RINK amide (to yield C-terminal amides) resins. The N-Fmoc protecting group was used throughout the syntheses, with *t*-butyl side chain protection on Ser, Thr, Glu, Asp and trityl protection on the side chains of Asn and Gln. Couplings were performed with HBTU as the coupling reagent. The phosphotyrosine was introduced as the protected phospho-tyrosine derivative, Fmoc-Tyr(OP(OMe)$_2$. Cleavage and deprotection of the phosphopeptides were carried out by treating the resin with a solution of TFA/Me_2S/TFMSA/*m*-cresol at 25 °C followed by a diethyl ether extraction protocol. Final purification was achieved by reverse phase HPLC to provide analytically pure peptides (>98%).

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6. This is in contrast to proteases where specificity and binding affinity is derived from the N- or C-terminal amino acids:

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9. (a) Zhang, A.-Y.; Davis, J. P.; Van Etten, R. L. *Biochemistry* **1992**, *31*, 1701. (b) The benzyoxyglycidyls and a number of other commercially available epoxides were inactive (>500 μM) against CD45 (data not shown).

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