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Phosphopeptide substrates and phosphonopeptide inhibitors of protein-tyrosine phosphatases

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Introduction

Protein-tyrosine phosphatases (PTPases) dephosphorylate phosphotyrosine residues in tyrosine kinases and their cellular substrates. Therefore, PTPase action is crucial for attenuating signals associted with Tyr phosphorylation. In this study we prepared substrates and inhibitors of PTPases based on the sequences of kinase and endogenous substrate phosphorylation sites. Corresponding phosphopeptides were synthesized for use as PTPase substrates, whereas inhibitors selected substrates were substituted at pTyr positions with phosphonomethylphenylalanine (Pmp), an unnatural, non-hydrolyzable amino acid analog in which the $>C\text{-}O\text{-}PO_3H_2$ moiety of pTyr is replaced by $>C\text{-}CH_2\text{-}PO_3H_2$.

Results and Discussion

Phosphopeptides were initially synthesized by solid-phase methods (DIPCDI/HOBt couplings in DMF) using Fmoc-pTyr with an unprotected phosphate side chain; PTPase inhibitors were synthesized similarly with Fmoc-Pmp [1]. In some cases synthetic products were relatively homogeneous, particularly when pTyr or Pmp was incorporated toward the amino-terminus of the peptide; crude products having greater homogeneity were obtained recently with protected synthons including Fmoc-pTyr$(OMe)_2$ [2] and Fmoc-Pmp$(OtBu)_2$ [3]. Phosphopeptides and phosphonopeptides were purified by HPLC, and gave the expected amino acid composition and FABMS values.

Known or putative phosphorylation sites of the insulin receptor (IR), PDGF receptor (PDGF-R), pp60$^{c\text{-}src}$, IRS-1 [4] and the polyomavirus transforming protein, middle t were synthesized. Whereas most of these sequences have only one Tyr residue, activation of the insulin receptor requires phosphorylation of three clustered Tyr residues [5]; all three monophosphopeptides were prepared. The phosphopeptides were used as substrates of PTPase 1B, a single catalytic domain PTPase with a wide tissue distribution [6]; rat PTPase 1B [7] was obtained by PCR and cloning the full-length cDNA into the bacterial expression vector, PKK233-2. Sequence specificity for peptide dephosphorylation was observed, with apparent K_m values ranging from $<2\ \mu M$ for the phospho-middle t sequence to >2 mM for pTyr itself (Table 1). K_m values were lower and specificity different

Table 1 *Sequences and potencies of PTPase substrates and inhibitors*

Name	Sequence/Structure	K_m or K_i (μM)
Phosphotyrosine and pY-peptides		
IR1155–1165(pY1158)	RDIpYETDYYRK	30
IR1155–1165(pY1162)	RDIYETDpYYRK	40
IR1155–1165(pY1163)	RDIYETDYpYRK	30
pp60$^{c\text{-}src}$(pY527)	TEPEpYQPGE	8
mPDGF-Rβ(pY719)	KDESIDpYVPMLDMKGD	8
middle t(pY298)	RENEpYMPMAPQIH	<2
IRS-l(pY608)	TDDGpYMPMSPGV	30
IRS-l(pY628)	GNGDpYMPMSPKS	100
phosphotyrosine	$NH_2CH(CO_2H)CH_2C_6H_4OPO_3H_2$	>2000
Phosphonomethylphenylalanine and Pmp-peptides		
IR1155–1165(L-Pmp1158)	RDI[L-Pmp]ETDYYRK	30
IR1155–1165(D-Pmp1158)	RDI[D-Pmp]ETDYYRK	30
IR1155–1165(D,L-Pmp3)	RDIPmpETDPmpPmpRK	<2
Pmp	$NH_2CH(CO_2H)CH_2C_6H_4CH_2PO_3H_2$	>500

than that observed with similar peptides and LAR (leukocyte antigen-related PTPase) [2], suggesting that substrate 'fingerprinting' might be useful for categorizing PTPases within families. Notably, with PTPase 1B the three insulin receptor monophospeptides exhibited similar K_m values in the 30 μM range, in contrast to results with LAR where regiospecificity was observed [2].

Representative phosphopeptide substrate sequences were prepared as phosphonopeptides for use as inhibitors. Pmp was synthesized chemically as a racemic mixture which was not resolved prior to peptide synthesis. Synthetic products were readily separated into two components by HPLC, corresponding to peptides having D- and L-Pmp [3]. Peptides containing L-pTyr and L-Pmp had similar affinities for PTPase 1B, suggesting that our inhibitor design strategy is appropriate (Table 1). Interestingly, the D-Pmp insulin receptor sequence had similar affinity for PTPase 1B. A related peptide, prepared with all three Tyr residues substituted with Pmp showed more potent inhibition (with 3 chiral centers this was a mixture of nine unresolved optical isomers).

We conclude that while side-chain unprotected Fmoc-pTyr and Fmoc-Pmp can be used to prepare phosphopeptides and phosphonopeptides by solid-phase synthesis, use of side-chain protected synthons results in cleaner products.

Synthetic phosphotyrosyl peptides corresponding to kinase and kinase substrate phosphorylation sites are substrates of PTPase 1B that exhibit sequence specificity. Phosphonomethylphenylalanyl peptides constitute a new class of compounds that potently inhibit PTPase activity. The Pmp-peptides studied appear to act as direct substrate mimics, as binding affinity closely matches that of the corresponding phosphopeptides and inhibition is competitive.

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