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Potent Inhibition of Insulin Receptor Dephosphorylation By a Hexamer Peptide Containing the Phosphotyrosyl Mimetic F_2Pmp

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Summary: Phosphonomethyl phenylalanine (Pmp) is a non-hydrolyzable phosphotyrosyl (pTyr) mimetic, which has been incorporated into eleven-mer Pmp-containing peptides that have previously been reported to competitively inhibit the protein-tyrosine phosphatases PTP1 and PTP 1B. We have recently shown that phosphonodifluoromethyl phenylalanine (F_2Pmp) is superior to Pmp as a pTyr mimetic in SH2 domain-binding peptides. Herein we find using the hexameric peptide sequence Ac-D-A-D-E-**X**-L-amide, where **X** = (D/L)-Pmp or L-F_2Pmp, that the half maximal inhibition values of these two peptides against PTP 1B-mediated dephosphorylation of autophosphorylated insulin receptor to be 200 μM and 100 nM, respectively. These data indicate that F_2Pmp induces a three orders of magnitude enhancement in affinity relative to Pmp, resulting in an exceptionally potent peptide-based PTP inhibitor. We conclude that F_2Pmp may be a generally useful tool in the preparation of selective, high affinity PTP inhibitors.

Aberrant cell signalling can cause or accentuate a variety of disease process including immune dysfunction, certain cancers and diabetes. For this reason cell signalling pathways have become targets for the development of new therapeutic agents (1, 2). Particularly important in growth factor and cytokine-mediated signalling is the strategic role of phosphotyrosyl (pTyr, **1**) residues, which appear to serve as molecular switches that can both activate and inactivate down stream signalling processes (3). Binding of ligands to the extracellular domain of growth factor receptors, induces autophosphorylation of their intracellular (cytosolic) domains and activates protein-tyrosine kinase (PTK) activity, resulting in endogenous substrate phosphorylation on specific tyrosyl residues and further signal transduction (4). The actions of PTKs are counterbalanced by protein-tyrosine phosphatases (PTPs) which hydrolyze pTyr phosphate esters (5), and which would conceptually be expected to act as inhibitory regulators of PTK-mediated signalling. However, mounting evidence suggests that PTPs may also be positive signal effectors in several systems (6). For example, PTPα, CD45 and $p80^{cdc25}$ are PTPs which can activate the PTKs, $p60^{src}$ (7) and $p56^{lck}$ (8) and the serine-threonine kinase $p34^{cdc2}$ (9)respectively, perhaps by

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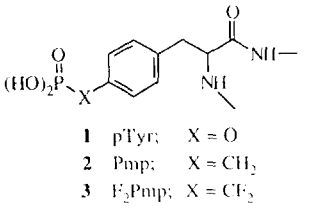

1 pTyr; X = O
2 Pmp; X = CH_2
3 F_2Pmp; X = CF_2

Ac-D-A-D-E-[(D/L)-Pmp]-L-amide
4

Ac-D-A-D-E-[(L)-F_2Pmp]-L-amide
5

Figure 1. Structures of pTyr, pTyr mimetics and inhibitor peptides.

dephosphorylating inhibitory pTyr residues. PTPs also appear to be required for the mitogenic effects of some cytokines, such as interleukin-4 (IL-4) (10) and for some interferons (11). A recent report that "Syp" (SH-PTP2) is a positive mediator of growth factor-stimulated mitogenic signal transduction in mammalian fibroblasts (12) adds further evidence that PTPs can upmodulate cell signalling. PTPs may also contribute directly to disease processes, as exemplified by the insulin receptor PTK, which is activated by autophosphorylation following binding of insulin to the extracellular ligand-binding domain, and where hydrolysis of these activating pTyr residues by PTPs could potentially exacerbate diabetic conditions (13).

In spite of the potential value which PTP inhibitors may present for the study of signal transduction pathways and for therapeutic intervention, relatively little has been reported on the development of such agents. Several metal-containing PTP inhibitors are known, including vanadate, oxovanadium complexes (14, 15) and gallium nitrate (16) as well as large, highly charged molecules such as suramin (17) and melittin (18). All of these agents would be expected to act in a fairly nonspecific fashion. The search for small molecule inhibitors has recently yielded the nitrosoamine-containing fermentation product "dephostatin" (19) and the irreversible suicide inhibitor, 4-difluoromethyl phenylphosphate (20), however both of these latter compounds could potentially generate highly toxic metabolites.

Another approach toward the design of PTP inhibitors relies on the replacement of pTyr residues in PTP peptide substrates with non-hydrolyzable phosphate mimetics. Phosphonic acids are isosteric with parent phosphates, yet are chemically and enzymatically resistant to P-C bond cleavage, making them valuable phosphate mimetics in a variety of biologically relevant contexts (21, 22). Phosphonomethyl phenylalanine (Pmp, **2**) is a phosphonate-based surrogate of pTyr in which the phosphate ester oxygen has been replaced by a methylene unit, and Pmp-containing peptides have previously been shown to act as competitive PTP inhibitors (23, 24). Pmp-bearing peptides also bind to src homology 2 (SH2) domains similar to the native pTyr-containing peptides, yet with reduced affinity (25). We recently reported the development of the Pmp derivative, phosphonodifluoromethyl phenylalanine (F_2Pmp, **3**) which bears two fluorines substituted at the alpha methylene (26-28). Fluorines have the double effect of lowering the ionization constants of the phosphonate hydroxyls (29) and introducing hydrogen bonding interactions similar to the parent oxygen-containing phosphate. Peptides containing this fluorinated analogue showed enhanced SH2 domain binding potency relative to peptides containing unsubstituted Pmp, with F_2Pmp-peptides being in some cases, equal or superior to the native pTyr-peptides in SH2 domain binding potency (30).

In this paper we examine whether F_2Pmp-containing peptides exhibit enhanced inhibitory potency in PTP assays relative to their Pmp counterparts. We have utilized PTP 1B preparations for this study, and have prepared peptides bearing the sequence, Ac-D-A-D-E-**X**-L-amide, (where **X** = (D/L)-Pmp **2** or (L)-F_2Pmp **3**), which has recently been shown to be an optimal hexamer substrate sequence for rat PTP1 (24).

Materials and Methods

Materials. All chemicals used were of the highest purity commercially available. Protein tyrosine phosphatase 1B (PTP 1B) was obtained from Upstate Biotechnology Inc., (Lake placid, NY) and wheat germ agglutinin (WGA)-coupled to agarose was purchased from Vector Laboratories, Inc. (Burlingame, CA).

Peptide Synthesis. The tyrosine phosphate mimicking amino acids, X = D/L-Pmp (**2**) or L-F_2Pmp (**3**) were incorporated into $EGFR_{988-993}$ segments, D-A-D-E-X-L, using solid-phase peptide synthesis with Fmoc chemistry. Amino acids, Fmoc-D/L-Pmp(O*t*Bu)$_2$-OH and Fmoc-L-F_2Pmp(OEt)$_2$-OH, were synthesized according to our earlier published methods (28, 31). Both peptides were prepared using PAL resin (32), DIPCDI/HOBT coupling reagents and 20% piperidine/DMF for Fmoc deprotection. The resin-bound protected peptides were acetylated with 4% 1-acetylimidazole/DMF. Finally, the peptide Ac-D-A-D-E-[(D/L)-Pmp]-L-amide (**4**) was obtained in one step by simultaneous cleavage from the resin and deprotection with TFA containing 5% each (v/v) of ethanedithiol, *m*-cresol, thioanisole and water. For synthesis of the L-F_2Pmp-containing analogue (**5**), the fully protected resin-bound peptide was first treated with the above TFA reagent to yield Ac-D-A-D-E-[L-F_2Pmp(OEt)$_2$]-L-amide. The ethyl protective groups were then removed from the F_2Pmp side chain with an TFA / TMS-triflate / ethanedithiol / *m*-cresol/dimethylsulfide reagent mixture (33) to yield the fully deprotected peptide. The peptides were purified to homogeneity by reverse phase HPLC (Vydac C_{18}). The diastereomeric mixture containing D/L-Pmp could not resolved, since it eluted as a single sharp peak on HPLC. All peptides provided FAB mass spectra and amino acid analyses consistent with their assigned structures.

Cell Line. The Chinese Hamster Ovary (CHO) cell line transfected with an expression plasmid encoding the normal human insulin receptor [CHO/HIRc] used in this study was a generous gift from Dr. Morris F. White, Joslin Diabetes Center, Boston, MA. The cells were maintained in F-12 medium containing 10% fetal bovine serum and were cultured to confluence.

Preparation of Triton X-100 Solubilized Membranes. Membranes were prepared from CHO/HIRc cells, essentially as described by Liotta et al.(34). In brief, cells were scraped off the dishes in an isotonic homogenization buffer that contained 10 mM HEPES, pH 7.5, 0.25 M sucrose, 5 mM EDTA, 20 µg/mL aprotinin, 10 µg/mL leupeptin, 0.2 mM phenylmethylsulfonylfluoride (PMSF), and pelleted by centrifugation at 300 x *g* for 10 min. The cell pellet was resuspended in homogenization buffer (43 x 10^6 cells/mL) and homogenized twice using a Polytron homogenizer (Brinkman) at a setting of 7, for 15 sec. each time. The homogenate was centrifuged at 12,000 x *g* for 20 min at 4 °C, and the pellet containing nuclei, debris and mitochondria was discarded. The supernatant was centrifuged at 100,000 x *g* for 60 min. at 4 °C, and the resulting crude membrane pellets were washed and frozen at -70 °C. When needed, the membrane pellet was resuspended in solubilization buffer containing 50 mM HEPES, pH 7.5, 0.25 M sucrose, 20 µg/mL aprotinin, 10 µg/mL leupeptin, 0.2 mM PMSF and 1% Triton X-100 (w/v) (3 to 5 mg protein/mL solubilization buffer). After a 30 min. incubation on ice with occasional stirring, the mixture was centrifuged at 100,000 x g for 60 min. at 4 °C, and the insoluble material discarded. Insulin receptors from solubilized membranes were purified by passing through WGA column following the method of Brillon et al.(35). The WGA eluate that contained purified receptors was divided into 100 µl aliquots and stored at -70 °C.

Assay of Insulin Receptor Dephosphorylation by Recombinant PTP 1B. ^{32}P-labeling of WGA purified insulin receptors and assay of PTP 1B activity were carried out as described previously

(34). In brief, ^{32}P-labeled autophosphorylated insulin receptors (10 μg/mL) were incubated with 0.5 μg/mL recombinant PTP 1B at 22 °C in a 100 μL reaction containing 50 mM HEPES, pH 7.5, 0.1 mg/mL BSA, 5 mM DTT, 5 mM EDTA, 0.05% Triton X-100, in the absence or the presence of various peptides at the indicated concentrations. The assay was terminated at various intervals by transferring an aliquot of the reaction mixture to a tube that contained 1 volume of 2-fold concentrated Laemmli sample buffer (36); samples were heated at 95 °C for 5 min. prior to electrophoresis in 7.5% SDS-polyacrylamide gels under reducing conditions. The ^{32}P remaining into the 95 kDa insulin receptor ß-subunit was quantified by Betagen counting of the fixed and dried gels.

Results and Discussion

Although development of PTP inhibitors has important implications for the study of signal transduction and possible therapeutic intervention, little has been done in this area. The majority of inhibitors presently known are either nonspecific or have inherent potential for chemical toxicity. It had previously been disclosed that Pmp-containing peptides can competitively inhibit PTPs, most probably by binding within the catalytic site as nonhydrolyzable substrate mimetics (23, 24). Our previous experience in SH2 domain binding peptides, which showed that F_2Pmp is superior to Pmp as a pTyr mimetic (30), led us to examine the efficacy of F_2Pmp relative to Pmp in PTP-directed peptides. Prior reports of Pmp-containing PTP inhibitory peptides utilizing 11-mer sequences, have indicated K_i values of 30 μM against PTP 1B for a peptide corresponding to the insulin receptor PTK sequence 1155-1165 (23), and 10 μM against rat PTP1 for a peptide corresponding to the sequence of $EGFR_{988-998}$ (24). The latter study showed that "Ac-D-A-D-E-**pY**-L-amide" is one of the optimum substrate sequences for rat PTP1, and we chose this 6-mer sequence as the basis for our study. Both of these works employed small peptide substrates, while our studies made use of whole protein receptor substrate in the inhibition assays. The F_2Pmp-containing peptide **5** was synthesized using enantiomerically pure L-F_2Pmp reagent (28). The Pmp-containing peptide **4** was prepared using racemic (D/L)-Pmp reagent (31), resulting in a mixture of L-Pmp and D-Pmp containing peptides, which could not be separated on HPLC. Since the D-Pmp residue is of the unnatural configuration, the mixture of D-Pmp and L-Pmp peptides would be expected to exhibit a net inhibitory potency less than a diastereomerically pure L-Pmp containing peptide.

The effect of these F_2Pmp- and Pmp-containing hexamer peptides on PTP 1B catalyzed insulin receptor dephosphorylation was examined. For our study we used intact phosphorylated insulin receptor as substrate, which is more physiologically relevant than short synthetic phosphopeptide substrates as used previously (23, 24). As shown in Figure 2, the L-F_2Pmp-containing peptide **5** exhibited half maximal inhibition at 100 nM while the (D/L)-Pmp-containing peptide **4** showed half maximal inhibition at 200 μM. Assuming negligible affinity for the D-Pmp-containing component of the peptide mixture, the L-Pmp-containing peptide would be expected to have a IC_{50} value of at least 100 μM. At 100 nM the L-F_2Pmp-containing peptide is 1000 times more potent than its unflourinated L-Pmp-containing counterpart, thus demonstrating that introduction of the F_2Pmp group results in a potent inhibitor of PTP 1B catalyzed insulin receptor dephosphorylation. The F_2Pmp moiety is therefore a valuable new motif for the preparation of PTP inhibitors. Our previous finding that F_2Pmp serves as a

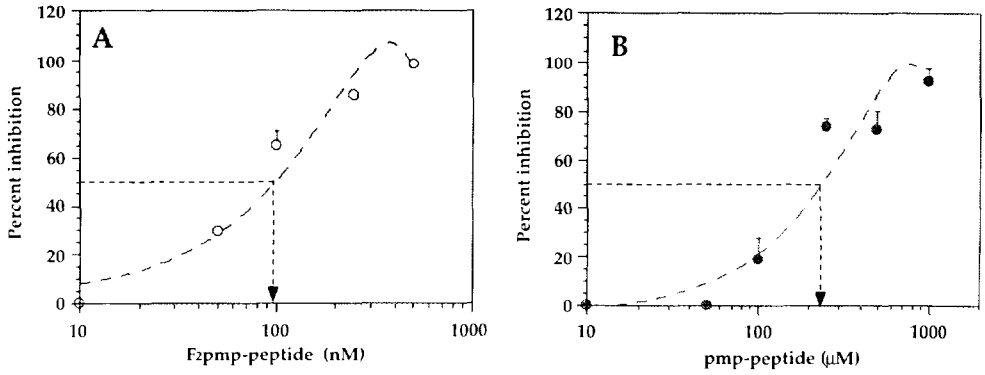

Figure 2. Effect of pmp and F_2pmp peptides on recombinant PTP 1B activity. ^{32}P-labeled insulin receptors were incubated with the recombinant protein tyrosine phosphatase, PTP 1B, in the absence or presence of various concentrations of F_2pmp-peptide **5** (A) or pmp-peptide **4** (B). The dephosphorylation reaction was carried out for 1 min. at 22 °C as described in "Materials and Methods". The activity of PTP 1B was in linear range under these assay conditions. Results are mean ± S.E. of two experiments performed in duplicate. Under identical assay conditions vanadate showed half maximal inhibition at a concentration of greater than 1 mM (data not shown).

non-hydrolyzable pTyr replacement in peptides which retain high affinity SH2 domain binding, is therefore extended to the field of PTP inhibitors. The apparent high affinity binding and selectivity of SH2 domain-binding phosphopeptides (37), contrasts with the observation that high affinity substrate recognition by PTPs may reside in residues to the N-terminal side of pTyr (24). It may therefore be possible to direct F_2Pmp-containing peptides against PTPs selectively by utilizing appropriate amino acid sequences. Additionally, to the extent that PTPs exhibit discrimination with respect to their substrate sequence (38) it may be possible to make inhibitors which are selective for individual PTPs. Such specific peptide inhibitors could not only be useful as tools for the isolation and characterization of physiologically relevant PTP's acting on specific phosphoprotein substrates, but could additionally find value as therapeutic agents.

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