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Negative Regulation of a Protein Tyrosine Phosphatase by Tyrosine Phosphorylation

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Reversible tyrosine phosphorylation is a major mechanism for cell signal transduction, and its dysregulation contributes to a vast range of diseases, including cancer. Protein tyrosine phosphatases (PTPs) are critical in opposing the actions of numerous protein tyrosine kinases.[1,2] Protein tyrosine phosphatases utilize a conserved active site triad of Cys, Arg, and Asp residues which are responsible for enzyme-catalyzed hydrolysis of phosphotyrosine-containing proteins.[1,2] The low molecular weight protein tyrosine phosphatase (LMW−PTP), a nonclassical PTP superfamily member, is known to be involved in modulating growth factor responses and reorganizing the cytoskeleton (Figure 1a).[3−5] A potential mechanism of regulation of the mammalian LMW−PTP is tyrosine phosphorylation. Two in vivo sites (Tyr-131 and Tyr-132) of LMW−PTP phosphorylation have been mapped, and these events have been proposed to be catalyzed by PDGFR (platelet-derived growth factor receptor) kinase, Src, or both.[6] Tyrosine phosphorylation of PTPases is difficult to study because of the intrinsic tendency of these enzymes to autodephosphorylate. Nevertheless, two groups have reported PTPase activation (2−25-fold) of LMW−PTP by tyrosine phosphorylation.[7,8] These studies were technically limited by the use of ATPγS-mediated phosphorylation of LMW−PTP to inhibit dephosphorylation, low stoichiometry of phosphorothioate incorporation, and application of mutagenesis to permit site-specificity of modification.

Here we have revisited the role of tyrosine phosphorylation of LMW−PTP by site-specific incorporation of the nonhydrolyzable pTyr analogues Pmp (phosphonomethylenephenylalanine). These analogues were introduced into human LMW−PTP by protein semisynthesis using expressed protein ligation, a strategy used previously in the context of analyzing tyrosine phosphorylation of SHP-1 and SHP-2.[9] In this approach, the N-terminal segment is produced as a recombinant thioester by fusion to an intein and then the C-terminal fragment is synthesized as an N-Cys-containing synthetic peptide. The two are linked via native chemical ligation (Figure 1b). To accommodate this method, a single residue (Gln-124) was replaced with Cys, which we showed was well-tolerated using standard site-directed mutagenesis (less than 2-fold difference in catalytic rates).[10] The LMW−PTP recombinant thioester fragment could be successfully ligated with N-Cys-containing synthetic peptides containing 0, 1, or 2 Pmp groups at the phosphorylation sites (Figure 1b). These semisynthetic proteins were purified to near homogeneity and refolded and concentrated to 1 mg/mL. Semisynthetic LMW−PTP protein produced in this fashion appeared nearly homogeneous on SDS−PAGE, MALDI-TOF spectra, and native gel (Figures S1 and S2).[10]

The unmodified protein (YY) showed phosphatase activity with *p*-nitrophenol phosphate (pNPP) substrate that was nearly identical with that of standard recombinant LMW−PTP (Figure S3a).[10] Initial PTPase assays of the four semisynthetic proteins (YY, Pmp131, Pmp132, and Pmp131/132) with pNPP as substrate revealed that Pmp131 and Pmp131/132 showed 2-fold weaker catalytic activity compared with that of YY and Pmp132 (Figure S3a).[10] Given previous reports of activation of LMW−PTPase by phosphorylation of Tyr-131, these results were unexpected. Since the catalytic differences we observed with pNPP substrate were rather modest, we considered the possibility that tyrosine phosphorylation might change cellular stability or localization. Microinjection experiments into REF52 cells indicated no significant differences in either the stability or cytoplasmic localization among the YY, Pmp131, and Pmp132 semisynthetic proteins (Figure S3b and S3c).[10] While these experiments do not rule out subtle changes in protein−protein interactions, they do argue against macroscopic changes in cellular location or protease sensitivity.

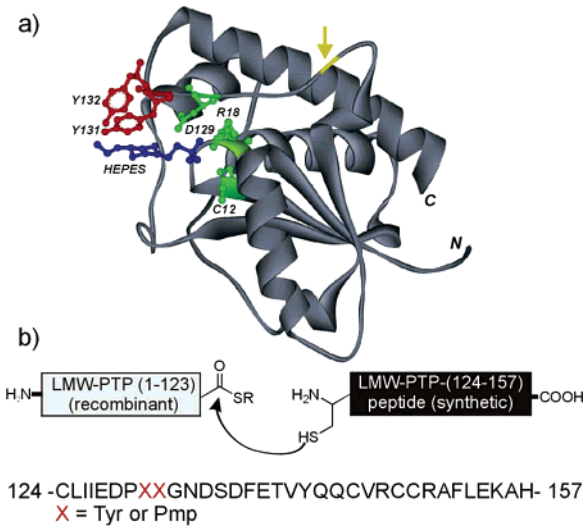

Figure 1. (a) The crystal structure of the bovine heart LMW−PTP with bound HEPES (blue).[12] Catalytic residues are highlighted in green, phosphorylation sites in red, and the junction between the recombinant and synthetic part is marked with a yellow arrow. (b) Generation of the semisynthetic LMW−PTPs.

To further investigate the effects on catalysis, a phosphotyrosine peptide derived from the PDGF receptor was examined as a substrate for the LMW−PTP semisynthetic proteins (Figure 2a). This phosphopeptide was designed based on the proposed regulatory role of LMW−PTP in dephosphorylating and down-regulating the PDGF receptor.[3,4] Unexpectedly, Pmp131, Pmp132, and Pmp131/132 LMW−PTP semisynthetic proteins showed marked decreases compared with YY in their efficiencies in dephosphorylating the PDGFR pTyr peptide. Rate reductions for Pmp131, Pmp132, and Pmp131/132 were 23-fold, 18-fold, and more than 45-fold down compared with YY, respectively (Figure 2a). Interestingly, the PTPase activity versus substrate concentration plot for YY showed partial saturation with an estimated K_m of approximately 300 μM. In contrast, the plots with Pmp131 and Pmp132 reactions showed no evidence of saturation up to 300 μM, suggesting a considerably higher K_m.

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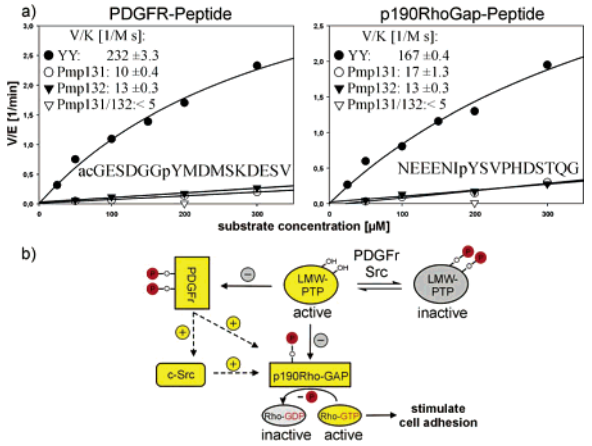

Figure 2. (a) Activity of semisynthetic LMW−PTPs versus peptide substrate concentrations. (b) Proposed model for the regulation of LMW−PTP by tyrosine phosphorylation.

To examine the generality of this finding, kinetics with the p190RhoGap-derived pTyr peptide were also investigated (Figure 2a). Another proposed function of LMW−PTP is to dephosphorylate tyrosine phosphorylated p190RhoGap, which is believed to be Src-phosphorylated on position 1105.[11] Kinetic results with the LMW−PTP semisynthetic proteins and the p190RhoGap-derived substrate phosphopeptide were rather similar to those with the PDGFR substrate peptide (Figure 2a). We observed about 10-fold rate reductions with Pmp131 and Pmp132 as catalysts compared with YY LMW−PTP when p190RhoGap phosphopeptide was used as substrate. Taken together, results of these kinetic studies strongly suggest that tyrosine phosphorylation of LMW−PTP at either or both of its in vivo sites of modification markedly down-regulates its phosphatase activity against physiologic targets.

In principle, the differences in catalytic efficiency could be related to the different leaving group behaviors of a Tyr (pK_a 10) versus that of nitrophenol (pK_a 7.5). To address this possibility, the phosphatase activities of the semisynthetic LMW−PTPs were examined with phenyl phosphate as substrate since phenol has a similar pK_a to the side chain of Tyr. The relative activities of YY, Pmp131, Pmp132, and Pmp131/132 were remarkably similar to those reactions with pNPP as substrate (Figure S3a).[10] These results establish that it is not leaving group ability that distinguishes the behavior of pNPP from phosphopeptide substrate. Rather, it is likely that the structural features of the substrate peptide's backbone or its side chains render PDGFR and p190RhoGap-derived substrates inferior with Pmp-modified LMW−PTP.

While the nature of LMW−PTP−phosphopeptide interaction has not been characterized in a high-resolution crystal structure, it is noteworthy that a mammalian LMW−PTP has been cocrystallized with a plausible substrate analogue HEPES (4-(2-hydroxyethyl)-1-piperazineethylsulfonate) molecule (Figure 1a).[12] This structure reveals the distal surface of the HEPES moiety in relatively close proximity to the surface Tyr-131 and Tyr-132 residues (3.5 and 3.4 Å, respectively). On the basis of this model, it can be surmised that placing phosphonates (or phosphates) on these Tyr side chains could interfere sterically or electronically with peptide−enzyme interactions.

Whatever its structural basis, inactivation of LMW−PTP by C-terminal tyrosine phosphorylation can be understood as strengthening the PDGFR−Src-p190RhoGap−cytoskeletal reorganization signaling axis. PDGF receptor and Src activation are believed to target p190RhoGap for tyrosine phosphorylation. LMW−PTP, which has been proposed to catalyze dephosphorylation of the PDGFR and Src-phosphorylated p190RhoGap, would be derailed by Tyr-131 or Tyr-132 modification. This organizationally simple model is satisfying in providing a straightforward way for PDGF receptor stimulation to remove a negative influence on its overall signaling impact (Figure 2b). It also represents the first example to our knowledge of a PTPase being inhibited by tyrosine phosphorylation.

It is noteworthy that the earlier studies[7,8] had reported that tyrosine phosphorylation of LMW−PTP was activating rather than inactivating as reported here. In addition to the technical difficulties described above, the earlier work relied primarily on pNPP as substrate, which as we show here behaves very differently from phosphopeptide. Distinctive regulation of a phosphatase toward different substrate classes has been observed previously with the Ser/Thr phosphatase calcineurin.[13] These LMW−PTP studies also point to the benefit of performing in vitro enzyme studies under conditions that most closely resemble natural systems.

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Supporting Information Available: Detailed experimental procedures, characterization of the semisynthetic proteins, kinetic and microscopy data. This material is available free of charge via the Internet at http://pubs.acs.org.

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