Published in final edited form as:

Semin Cell Dev Biol. 2015 January ; 0: 66–72. doi:10.1016/j.semcdb.2014.09.021.

Mining the Function of Protein Tyrosine Phosphatases in Health and Disease

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Abstract

Protein tyrosine phosphatases (PTPs) play a crucial role in the regulation of human health and it is now clear that PTP dysfunction is causal to a variety of human diseases. Research in the PTP field has accelerated dramatically over the last decade fueled by cutting-edge technologies in genomic and proteomic techniques. This system-wide non-biased approach when applied to the discovery of PTP function has led to the elucidation of new and unanticipated roles for the PTPs. These discoveries, driven by genomic and proteomic approaches, have uncovered novel PTP findings that range from those that describe fundamental cell signaling mechanisms to implications for PTPs as novel therapeutic targets for the treatment of human disease. This review will discuss how new PTP functions have been uncovered through studies that have utilized genomic and proteomic technologies and strategies.

Keywords

protein tyrosine phosphatases; phosphorylation; substrates; signal transduction; proteomics; genomics

Introduction

Protein tyrosyl phosphorylation is fundamental to the maintenance of numerous cellular functions including gene expression, cell growth, differentiation, migration, adhesion and apoptosis [1]. The net level of cellular protein tyrosyl phosphorylation is balanced by the opposing actions of both protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The importance of maintaining homeostatic control of cellular tyrosyl phosphorylation is exemplified by the observation that dysregulation of these processes

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often results in the development of diverse pathophysiological conditions that can include cancer, metabolic, neuronal and immunological diseases [2].

There are a number of excellent reviews that have been published on the PTPs. There are reviews that cover the PTP field from a historical perspective [3]. Whilst others have focused on the role of PTPs in hereditary human diseases [2] as well as viral and bacterial pathogenesis [4]. Comprehensive reviews also exist on PTPs in human cancer [5] and the involvement of lipid-phosphatases in human disease [6]. RPTPs and the biological insight uncovered by the solution of several RPTP crystal structures have also been topics of discussion [7, 8]. We direct the reader to these sources for more comprehensive discussions since this review will focus largely on the application of non-biased screens as a discovery tool for PTP function.

In many regards PTP research continues to progress rapidly as a function of the emergence of new technological advances in the biological sciences. These include in more recent years the explosion of system-wide non-biased approaches using genomic and proteomic strategies. The goal of this review will be to focus on some of the more recent discoveries in the PTP field that have utilized approaches in genomics and proteomics to uncover PTP function. The application of system-wide non-biased strategies to PTP research has revealed new functions of PTPs in a variety of physiological and pathophysiological settings that highlight the critical role played by these enzymes in cell signaling. This review will focus on the discoveries of PTP function that utilize these types of system-wide non-biased strategies.

The PTP Superfamily

The classical PTPs include 16 non-transmembrane PTPs and 21 receptor-like PTPs (RPTPs) all of which contain a common conserved core catalytic PTP domain defined by the signature motif C(X)₅R [9] (Fig. 1). The classical PTPs are classified into 17 PTP subtypes: nine non-transmembrane (NT1 to NT9) and eight receptor types (R1/R6, R2a, R2b, R3, R4, R5, R7, and R8) (Fig. 1) [9]. The non-transmembrane PTPs exhibit a variety of non-catalytic domains that mediate functions such as protein-protein interactions (e.g. SH2 and PDZ domains), lipid-binding domains (e.g. FERM) and sub-cellular targeting motifs (e.g. PTP-1B C-terminal ER targeting motif). These non-catalytic domains also play key roles in the regulation of phosphatase activity by coordinating intramolecular interactions that engage in conformational changes that either activate or inactivate PTP catalysis. Furthermore, the post-translational modifications of PTPs, such as proteolysis, phosphorylation, and oxidation, also participate in sub-cellular localization, protein-protein interactions, regulation of catalytic activity, and protein stability [10-13].

RPTPs comprise of variable extracellular domains, a single transmembrane domain, and a cytoplasmic domain containing two, and in some cases, a single PTP domain [14, 15]. Since their extracellular domains are similar in structure to the extracellular domains found in cell adhesion molecules RPTPs appear to be involved in cell-cell and cell-matrix communications [7]. Typically, ligand binding to the extracellular domain of an RPTP induces dimerization that leads to the inhibition of its catalytic activity [16-19]. In the

dimeric state, reciprocal inhibition of the catalytically competent proximal D1 PTP domain occurs whereby the “wedge motif” of one D1 domain occludes the active site of the opposing D1 PTP domain in the dimer [15, 19]. The distal, D2 PTP domain is catalytically inactive although it provides important regulatory functions such as stabilizing substrate interactions, mediating protein-protein interactions and facilitating RPTP dimerization [17, 20-23]. Although this mechanism of dimerized-induced PTP inhibition exists for some RPTPs it is still unclear as to how conserved this mode of PTP operation is amongst the entire RPTP family.

The dual-specificity phosphatase (DUSP) family of PTPs comprises of PTPs that dephosphorylate both lipid and protein substrates. The more extensively characterized sub-family of DUSPs are those that catalyze the dephosphorylation of the mitogen-activated protein kinase (MAPK) phosphatases (MKPs). The MKP family comprises of ten catalytically active enzymes that share a common PTP catalytic domain at their carboxyl terminus and a non-catalytic regulatory domain at the amino terminus (Fig. 2) [24-26]. The amino-terminus non-catalytic domain contains a cdc25 homology (CH2) domain and a kinase interaction motif that binds directly to the MAPKs in order to coordinate MKP-MAPK substrate dephosphorylation [27, 28]. These enzymes are classified based upon substrate specificity, sequence similarity and sub-cellular distribution into three groups namely; Type I, Type II and Type III [29, 30]. Type I MKPs consist of MKP-1, MKP-2, PAC1 and hVH3. This group of MKPs primarily localizes to the nuclear compartment and is induced by many stimuli that activate MAPKs. Type II MKPs, which selectively dephosphorylate ERK, include MKP-3, MKP-X and MKP-4 and they are localized to the cytoplasm. Type III consists of MKP-5, MKP-7, and M3/6, which shuttle between the cytoplasm and nucleus. They selectively dephosphorylate JNK and p38 MAPK but exhibit much less activity towards ERK1/2 [31]. In addition to the classical DUSPs there are also 16 members of the atypical DUSPs that lack the NH2-terminal CH2 domain found in the MKPs but consist of the DUSP catalytic domain (Fig. 2) [32].

New approaches to uncovering PTP function

The following sections will highlight how PTPs have been implicated in various processes and their function further realized through the use of unbiased system-wide screening strategies in genomics and proteomics. These approaches include the use of siRNA/shRNA and phosphoproteomics screens coupled with PTP-specific technologies such as substrate trapping for the identification of PTP substrates.

PTP function revealed by siRNA and shRNA screening

Genome-wide siRNA/shRNA screening has proven to be a very powerful tool for the discovery of new gene functions. Recently, these applications have been used to identify new and novel functions of PTPs. One of the major advantages of siRNA/shRNA screening largely revolves around the ability to design strategies that uncover functional effects of genes in an unbiased and system-wide manner. High-throughput screening at the genome-wide level has been performed based on the outcome of specific cellular phenotypes that include cell migration, apoptosis and proliferation in a variety of cell types including

epithelial cells, endothelial cells and various cancer cell lines. Collectively, the use of siRNA/shRNA approaches has been a successful strategy for uncovering new PTP functions.

Loss-of-function strategies using siRNA/shRNA approaches have focused predominantly on the identification of genes that have specific cellular phenotypes such as cell migration, apoptosis or proliferation. In contrast, others approaches using siRNA/shRNA screens have employed strategies in which a specific sub-family of genes have been selectively targeted and the analysis of the outcome broad in order capture new functions for the targeted genes (Fig. 3). In addition, siRNA/shRNA knockdown approaches have been coupled with other methodologies that further enhance the assignment of gene functionality to a specific molecular mechanism, as is the case with the application of substrate-trapping approaches for the PTPs (Fig. 3).

Using a siRNA library targeting 1,081 human genes Simpson et al identified genes that were involved in epithelial cell migration using MCF10A human breast epithelial cells [33]. Of the numerous genes identified several PTPs such as PTPRO, DUSP18, and DUSP24 were found to be involved in either positively or negatively regulating epithelial cell migration. A customized siRNA human kinase library targeted 650 genes, with the phosphatase library targeting 222 genes, which was used to identify new regulators of apoptosis and chemoresistance in cancer cell lines [34]. Of the genes screened, several PTPs were found to be either upregulated or downregulated in chemosensitive states. These included DUSP-5, INPP5A, MTMR7, PPP6C, and PSPH. The SH2 domain-containing tyrosine phosphatase-1 (SHP-1) was also identified as a potential synthetic lethal partner of the DNA repair protein polynucleotide kinase/phosphatase using a “druggable” genome siRNA library that comprises of 205 phosphatases, 696 kinases, 490 G-protein-coupled receptors and 5,570 uncategorized proteins [35].

siRNA screening approaches of PTPs have also been coupled with other techniques such as the quantitative assessment of differential levels of phosphotyrosyl content in siRNA/shRNA-treated cells in order to facilitate the identification of novel PTP substrates. For example, a phosphatase family siRNA library that contained 254 human siRNAs targeted to protein, lipid, and carbohydrate phosphatases was used to discover that PTPN12 negatively regulates tyrosine phosphorylation of TrkB and BDNF-TrkB mediated neurite outgrowth [36]. A smaller scale siRNA screen against 43 human PTPs revealed that PTPN9 targets ErbB2 and the epidermal growth factor receptor (EGFR) as its substrates in a breast cancer cell line [37]. Another siRNA human library targeting 39 PTPs was used to identify PTPRK and PTPRJ/DEP-1 as an EGFR-targeting phosphatase. The later finding further suggested that DEP-1 played a role in EGFR endocytosis [38]. A limited RNAi screen targeted against human classical PTPs revealed that PTP1B regulates IFN-alpha/beta receptor chain 1 (IFNAR1) endocytosis and thereby IFN γ signaling [39]. A genome-wide siRNA screen to identify molecules involved in autophagy uncovered a novel pathway that implicated PTP σ in the regulation of autophagy [40].

siRNA screens can also be coupled with other non-biased approaches to achieve more selective assignment of PTP function. A study from this laboratory using siRNA screen

against 12 human RPTPs discovered that PTPRF directly dephosphorylates phospho-tyrosyl 930 on EphA2 and negatively regulates EphA2-mediated cell migration [41]. In this screen siRNA was used to knockdown RPTPs followed by counter-screening against an array that represented up to 40 human receptor tyrosine kinases (RTKs). By comparing the degree of hyper tyrosyl phosphorylation on each of the RTKs between control and RPTP knockdown cells a fingerprint of RPTP-RTK associations at the level of tyrosyl phosphorylation was established. These experiments suggested that RPTPs have the capacity to target a unique sub-set of RTKs. Moreover, as in the case of PTPRF site-selective RTK dephosphorylation can be achieved [41].

Although siRNA screening has been widely used to identify new functions for genes, the siRNA method has limitations that include primarily the transient nature of gene knockdown. To overcome this limitation virus-based shRNA screening has been developed. Infection of virus encoding for shRNA dramatically enhances the efficiency of gene delivery into a wide variety of cell lines including primary cells. Furthermore, integration of viral shRNA into the host cell results in the stable knockdown of the target gene. A study by Lin et al used a retrovirus-based shRNA approach that represented four unique shRNA's per PTP grouped into 25 pools according to their structural similarity to the corresponding target PTP. The results of this screen identified PTPN23 as a novel regulator of cell invasion in mammary epithelial cells [42]. To identify novel biomarkers for early diagnosis of oral cancer, lentiviral shRNA targeting 1,236 genes including 737 kinases, 209 phosphatases and 30 genes with dual function revealed a total of 50 candidate genes, for which more than 90% represented effects on growth inhibition in human oral squamous cancer HSC-3 cells [43]. Of the genes identified two PTPs including PTEN and DUSP18 were found to inhibit cell growth. The notion that PTPs often serve as negative regulators of cell signaling prompted an shRNA screen of the PTP family to identify PTPs that could potentially serve as tumor suppressor genes. Using the mammary epithelial cell lines MCF10A and HMLE Wang et al identified PTPN14 in a shRNA screen as gene that potently induced anchorage-independent growth in soft agar [44]. Through mass spectrometry, YAP1 was identified to interact with PTPN14, and this interaction facilitated the retention of YAP1 to the cytoplasm. Given that YAP1 serves as a key oncogene in the Hippo pathway and that PTPN14 attenuates its function these data revealed the unexpected finding that PTPN14 participates as a novel tumor suppressor gene [44].

Proteomic applications to the discovery of PTP substrates

In order to understand the precise molecular pathways engaged by PTPs the identification of their substrates is essential. As discussed, the combination of knockdown approaches of the PTPs with proteomic techniques has proven to be a powerful strategy towards uncovering PTP function. In this regard, the use of loss-of-function (siRNA/shRNA) screening approaches and specifically, phosphoproteomics has been leveraged to identify PTP substrates. This section will discuss recent findings in PTP function that have utilized these methodologies to define PTP function (Fig. 3).

Proteomics: Substrate-trapping

The catalytic domain of the classical PTPs contains about 280 amino acids and comprises 10 conserved motifs that define substrate recognition and catalysis [9, 45]. Among these motifs, HC-(X₅)-R, known as the 'PTP signature motif' defines the PTP active site that catalyzes phosphotyrosyl dephosphorylation. Two additional motifs, the WPD (Trp-Pro-Asp) loop and the Q loop, are also important for substrate binding and catalysis. We direct the reader to more in-depth reviews on the molecular mechanisms of PTP catalysis [46]. Replacement of the active site cysteine by a serine (CS) leads to a dramatic loss of phosphatase activity [47]. The mutation of aspartate in the WPD loop to alanine (DA) decreases the substrate turnover (k_{cat}) without affecting the dissociation constant (K_m) of its substrate [47]. As such, the combined substrate-trapping mutant (CS/DA), in many but not all cases, provides an effective PTP-substrate affinity than either mutant alone [48]. Although, certainly each of the individual mutations, either CS or DA, can be utilized as a substrate-trapping PTP mutants by themselves. In addition, glutamate to alanine (QA) in the Q loop in context of the DA mutant (DA/QA) has been reported and this mutant also exerts enzyme-substrate complex stability [49, 50]. By implementing the substrate-trapping strategy, many PTP substrates have been identified. For example, EGFR [47, 51, 52] and Janus kinases (JAKs) [53, 54] were identified as the substrates for PTP1B. We identified the major vault protein (MVP) as a substrate of the SH2 domain-containing protein tyrosine phosphatase (Shp2) [55]. In this approach, cell lysates from human fibroblasts were substrate-trapped using Shp2 C459S or D425A mutants. Proteins in this complex were identified by matrix-assisted laser, desorption ionization by time of flight mass spectroscopy (MALDI-TOF) [55].

Phosphotyrosyl enrichment strategies

Given that PTPs signal through tyrosyl dephosphorylation, methodologies that are capable of differentiating between site-selective changes in phosphotyrosyl content in a given biological system offer valuable tools in which to uncover the molecular mechanisms engaged by PTPs. To this end, proteomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become a dominant technology for defining and understanding the key roles played by signaling molecules that regulate tyrosine phosphorylation. 7

A key step during phospho-tyrosine proteomic analysis with MS is enrichment and concentration of phosphopeptides prior to MS analysis [56-58]. Immobilized metal affinity chromatography (IMAC) is a chromatographic technique for phosphopeptide enrichment based on chelation between negatively charged phosphate groups on tyrosine with ions, such as Fe(III) or Ga(III), in solid chromatographic supports [59-61]. Although IMAC is a cost-effective, rapid, and efficient method to purify large quantities of protein, some negatively charged amino acids (cysteine, aspartic acid, and glutamic acid) can participate in binding to metal ions. IMAC also can induce the degradation of side chains of protein backbones by reactive radical species, which results from a metal-catalyzed oxidative process [62, 63]. Another chromatographic strategy for phosphopeptide enrichment is TiO₂ chromatography [64]. The selective phosphopeptide binding properties of TiO₂ resin results in different selectivity and preferential enrichment of multiply phosphorylated peptides compared to immobilized chelated metal ions (IMAC) [65-67]. For example, drosophila Ptp61F (the

ortholog of mammalian PTP1B and T Cell-PTP) was found following a phosphoproteomic screen to regulate phosphorylation of Stat92E (pY704), paxillin (pY48), and Abi (pY248) [68]. Furthermore, increased tyrosine phosphorylation of acyl-CoA binding protein (pY29), cortactin (pY334) and insulin receptor (pY1193 and pY1197) were identified from PTP-1B knockout mouse liver and fibroblasts using TiO₂ chromatography [69, 70].

Another tool to enrich for low abundance tyrosine phosphorylated proteins is the approach of immunoprecipitation with anti-phosphotyrosine antibodies [71, 72]. A global quantitative proteomic analysis has been applied after immunoprecipitation with anti-phosphotyrosine antibodies. Using this method, STAT3 (pY705) and p130Cas (pY128) were identified as substrates for PTPRT [73] and PTPN14 [74], respectively, in colorectal cancers. Moreover, it has been reported that Src (pY530) and Cdk1 (pY15), the auto-inhibitory sites of each kinase, were dephosphorylated by Shp2 in lung tumor tissue using similar phosphotyrosyl enrichment approaches [75].

Recently, in an attempt to identify targets for the mutation in Shp2 that causes congenital heart defects in Noonan syndrome (NS), we performed a phosphotyrosine proteomic screen in the heart of mice harboring the Noonan syndrome (NS)-associated Shp2 mutant (*Shp2^{D61G}*) [76]. Heart tissue isolated from wild type and NS mice were digested, immunoprecipitated with anti-phosphotyrosine antibodies, and subjected to LC-tandem MS (MS/MS) analysis. The relative abundance of tyrosyl phosphorylated proteins was assessed by quantitating the frequency of phosphopeptides derived from wild type and NS mice. We identified an approximately equal profile of differentially hypo- and hyper-tyrosyl phosphorylated peptides in the hearts of NS mice as compared with wild type controls [76] (Fig. 4). The limited number of differentially tyrosyl phosphorylated peptides suggests that the NS-associated Shp2 mutant induces a discrete influence on global tyrosyl phosphorylation levels in the heart. Furthermore, we also performed phosphoproteomic analysis from zebrafish embryos overexpressing NS-Shp2 (*Shp2^{D61G}*) or NSML-Shp2 (Noonan syndrome with multiple lentigines, formerly LEOPARD syndrome; *Shp2^{A462T}*). Remarkably, using phosphoproteomic approaches we identified protein zero-related (PZR), a transmembrane glycoprotein [77, 78], as a major hypertyrosyl phosphorylated protein in both mouse and zebrafish models of NS and NSML. Since NS-Shp2 and NSML-Shp2 show opposite catalytic activity but cause similar developmental disorders, these results suggest that PZR functions as a “phosphatase independent” target of Shp2 to mediate both NS and NSML pathogenesis [76]. Although phosphoproteomic analyses has the capacity to identify novel PTP targets as a result of differential tyrosyl phosphorylation levels in either PTP gain-of or loss-of-function scenarios several limitations remain. For example, it is difficult to determine if a phosphorylation change on a particular target protein is the direct or indirect effect of PTP activity or a subsequent downstream event. Therefore, follow-up biochemical approaches must be applied for the assignment of a *bona fide* PTP substrates to be defined [46, 79].

Conclusion

Over the last decade the biology of PTPs has developed rapidly due in part to the implementation of both genomic and proteomic approaches that allow for non-biased

system-wide query of the involvement of PTPs in a variety of different cellular systems and disease states. The continued integration of these approaches towards answering PTP-related questions should further our knowledge of PTP function in general and more specifically allow for the assignment of PTPs to novel signaling pathways.

Acknowledgements

A.M.B. was supported by R01 GM099801 and DK75576. A.L. was supported by the Brown Coxe Postdoctoral Fellowship and H. L. was the recipient of a Leslie H. Warner Postdoctoral Fellowship.

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Fig. 2. The dual-specificity phosphatase (DUSP) family
Schematic of the dual specificity phosphatases, which are comprised of the sub-family of MAP kinase phosphatases (MKPs) and atypical DUSPs.

Fig. 3. Schematic representation of approaches to identify PTP function and substrate
siRNA or shRNA can be selected according to the desired experimental situation. Substrates of PTPs can be identified using proteomic methods.

Fig. 4. Schematic of phosphoproteomic analysis of differentially tyrosyl phosphorylated proteins in mouse models of disease

Proteomic approaches can be used to differentially determine relative levels of tyrosyl phosphorylated proteins which can be analyzed by enrichment with anti-phosphotyrosine antibodies. A combination of biochemical approaches can be applied in order to validate protein targets.