REVIEW ARTICLE

Protein tyrosine phosphatase function: the substrate perspective

Tony TIGANIS* and Anton M. BENNETT†[1]

*Department of Biochemistry and Molecular Biology, Monash University, Victoria 3800, Australia, and †Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520, U.S.A.

It is now well established that the members of the PTP (protein tyrosine phosphatase) superfamily play critical roles in fundamental biological processes. Although there has been much progress in defining the function of PTPs, the task of identifying substrates for these enzymes still presents a challenge. Many PTPs have yet to have their physiological substrates identified. The focus of this review will be on the current state of knowledge of PTP substrates and the approaches used to identify them. We propose experimental criteria that should be satisfied in order to rigorously assign PTP substrates as *bona fide*. Finally, the progress that has been made in defining the biological roles of PTPs through the identification of their substrates will be discussed.

Key words: protein tyrosine phosphatase (PTP), substrate identification, substrate-trapping, tyrosine phosphorylation.

INTRODUCTION

The cellular equilibrium of protein tyrosine phosphorylation is achieved through the actions of PTKs (protein tyrosine kinases) and PTPs (protein tyrosine phosphatases). The realization that disrupting the equilibrium of cellular tyrosine phosphorylation causes a plethora of human diseases vividly exemplifies the importance of tightly controlling the activities of both PTKs and PTPs. Historically, our knowledge of the molecular actions of PTKs, PTK targets and their involvement in normal cell signalling and human disease has greatly outpaced that of PTPs. In part, this is attributed to the fact that PTKs were discovered almost a decade before PTPs, but in addition, as will be discussed, PTPs have unique issues and challenges.

PTPs are defined by a unique signature motif $HC(X)_5R$ that can be grouped into two general categories. The first group is the tyrosine-specific PTPs that dephosphorylate protein substrates on tyrosine. Tyrosine-specific PTPs comprise receptor-like PTPs and non-transmembrane PTPs (Figure 1). The second group is the DSPs (dual-specificity phosphatases) that dephosphorylate protein substrates on tyrosine, serine and threonine residues, as well as lipid substrates. This review will focus on the role of the non-transmembrane tyrosine-specific PTPs (Figure 1) and the role their substrates play in normal cellular signalling and, in some cases, human disease. It is not the intention here to generate a comprehensive listing of non-transmembrane PTPs and the signalling pathways for which they have been implicated in regulating. There are already a number of outstanding reviews that cover these areas and we direct the reader to those sources (see [1–4]). Instead, we will attempt to highlight, using selected examples, PTPs that have had substrates identified for them and where the function of these substrates provides insight into the biology of the PTP.

In order to define the molecular mechanism of action for the PTPs, one of the major questions to be addressed is the identity of the substrates that these enzymes dephosphorylate. The approaches for defining the function of PTPs have relied upon a combination of biochemical and genetic techniques. When applied in an integrative manner, these approaches have provided a wealth of functional information about PTPs (see reviews [1–4]). Notably, a subfamily of the DSPs called the MKPs [MAPK (mitogen-activated protein kinase) phosphatases] has been found to dephosphorylate MAPKs on both tyrosine and threonine residues within the activation loop of the kinase. The MKPs exhibit different specificities towards the various MAPKs and therefore function as critical negative regulators of MAPK-mediated signalling in a variety of biological processes (reviewed in [5,6]). The MKPs therefore serve to illustrate the successes of PTP substrate identification.

In contrast with the DSPs, the identification of substrates for the non-transmembrane tyrosine-specific PTPs (Figure 1) has been much more problematic. As we will discuss, these hurdles, although challenging, are not insurmountable. Now that all of the genes that comprise the PTP superfamily have been identified [1], attention to the function of the PTPs has intensified. Hence, an appreciation of the major issues that exist for defining PTP function is important. In this respect, we have sought to direct our attention towards two major challenges that currently exist in defining PTP function: (i) the identification of PTP substrates, and (ii) linking PTP substrates with the biology of the PTPs. Before

Abbreviations used: AP-1, activator protein 1; BCR, B-cell receptor; BIT, brain immunoglobulin-like molecule with tyrosine-based activation motifs; Cbp, C-terminal Src kinase-binding protein; CSF-1, colony-stimulating factor 1; CSK, C-terminal Src kinase; DSP, dual-specificity phosphatase; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; GAP, GTPase-activating protein; Gab1, Grb2-associated binder-1; HGF, hepatocyte growth factor; IFN, interferon; IGF-1, insulin-like growth factor 1; IR, insulin receptor; IRS, IR substrate; JAK, Janus kinase; KIM, kinase-interacting motif; LYP, lymphoid phosphatase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MKP, MAPK phosphatase; NFAT, nuclear factor of activated T-cells; NS, Noonan syndrome; NSF, *N*-ethylmaleimide-sensitive factor; PAG, phosphoprotein associated with glycosphingolipid-enriched membrane microdomains; PDGFR, platelet-derived growth factor receptor; PEP, PEST (Pro-Glu-Ser-Thr) domain phosphatase; PI3K, phosphoinositide 3-kinase; PIR-B, paired immunoglobulin-like receptor B; PSTPIP, proline/serine/threonine-phosphatase-interacting protein; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; RNAi, RNA interference; SFK, Src family kinase; SH, Src homology; SHP, SH2-domain-containing protein tyrosine phosphatase; SNARE, NSF-attachment protein receptor; SNP, single-nucleotide polymorphism; STAT, signal transducer and activator of transcription; STEP, striatal-enriched PTP; TCPTP, T-cell PTP; TCR, T-cell receptor; WASP, Wiskott–Aldrich syndrome protein; ZAP-70, ζ-chain-associated protein kinase of 70 kDa.

[1] To whom correspondence should be addressed (email anton.bennett@yale.edu).

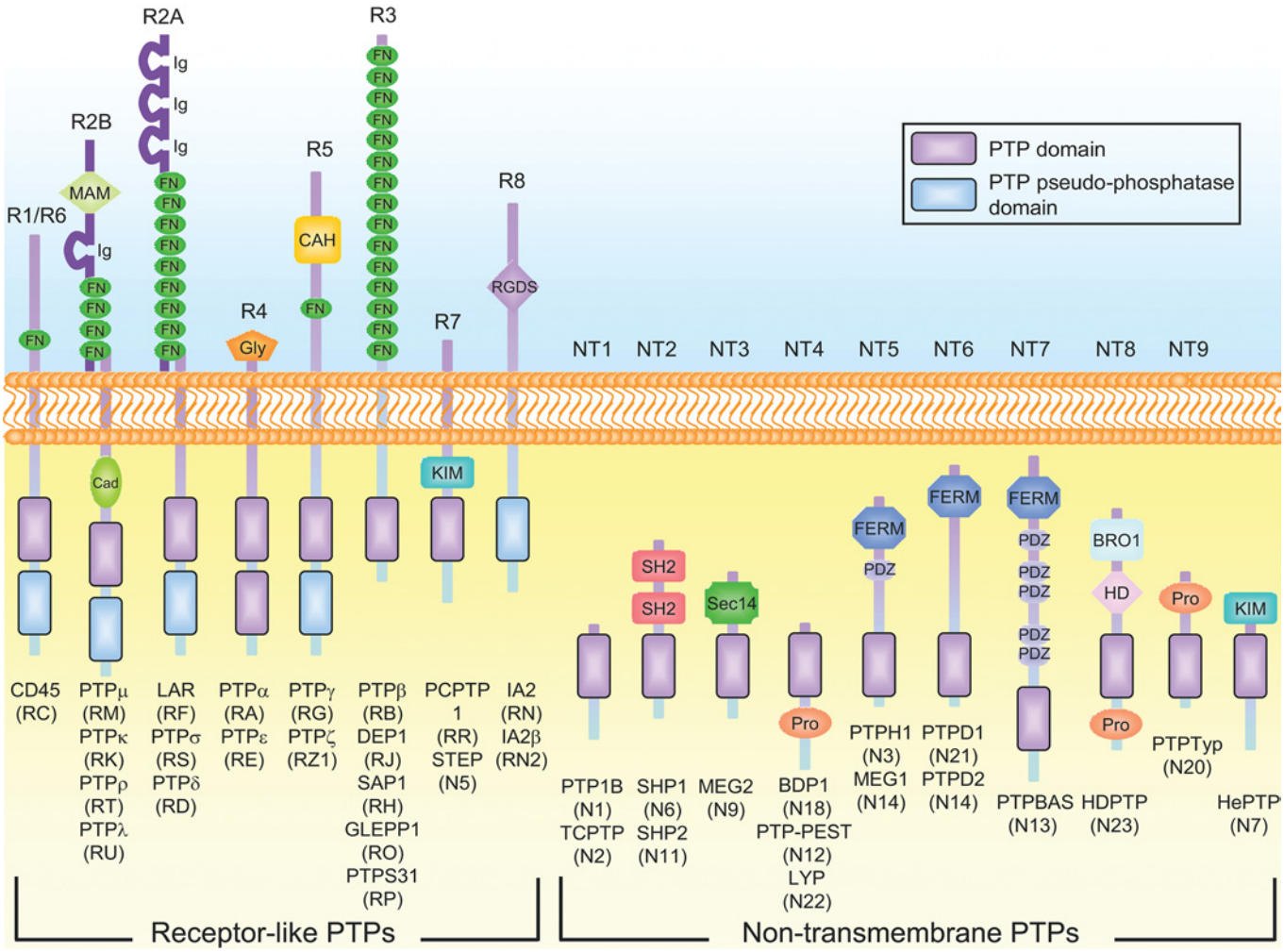

Figure 1 Structure of classical PTPs

Schematic representation of the classical PTPs grouped as either receptor-like PTPs or non-transmembrane PTPs which contain various functional domains [BRO-1, BRO-1 homology; CAH, carbonic anhydrase-like; Cad, cadherin-like juxtamembrane sequence; FERM, FERM (4.1/ezrin/radixin/moesin) domain; FN, fibronectin type III-like domain; Gly, glycosylated; HD, histidine domain; Ig, immunoglobulin domain; KIM, kinase-interacting motif; MAM, mephrin/A5/μ domain; Pro, proline-rich; RGDS, RGDS-adhesion recognition motif; SEC14, SEC14/cellular retinaldehyde-binding protein-like; SH2, Src homology 2]. Some of the receptor-like PTPs contain a membrane proximal PTP domain that is catalytically active and a membrane-distal PTP domain (PTP pseudo-phosphatase domain) that has residual activity. The non-transmembrane PTPs all contain a single PTP domain.

tackling these issues, a brief overview of the principles of PTP catalysis and substrate recognition will be discussed.

SUBSTRATE RECOGNITION BY PTPs

Although initially viewed as broad-specificity 'housekeeping' enzymes, it is now realized that tyrosine-specific PTPs are highly selective enzymes that exhibit striking specificity for protein substrates in a cellular context. The ability of PTP family members to differentiate between individual substrates can be attributed to the inherent specificity within the PTP catalytic domain. An additional level of substrate specificity is facilitated further through the non-catalytic N- and C-terminal segments. These non-catalytic domains target the PTP to specific subcellular locales for substrate recognition and substrate binding.

The catalytic domains of classical PTPs contain approx. 280 residues and comprise 22 invariant and 42 highly conserved residues that fall within ten consensus motifs [2]. These motifs contribute to conserved folds or can otherwise be involved in substrate recognition and catalysis (for more in-depth reviews, see [2,7]). The active-site sequence $HC(X_5)R$ defines the PTP family and this sequence is referred to as the 'PTP signature motif'. Residues in this motif form the phosphate-binding loop which is located at the base of the active-site cleft. The cysteine and arginine residues in the PTP signature motif are essential for catalytic activity. The cysteine residue acts in the first step of catalysis wherein the sulfur atom of the thiolate group serves as a nucleophile and attacks the substrate phosphate. The arginine residue contributes to substrate binding and stabilizes the cysteine-phosphate intermediate. Another important motif integral to PTP catalysis is the WPD (Trp-Pro-Asp) loop, which becomes displaced by several angstroms [8–12 Å (1 Å = 0.1 nm)] upon substrate binding and closes around the side chain of the phosphotyrosine residue. This conformational change positions the invariant aspartate residue (Asp^{181} in PTP1B) in the WPD loop in a position that allows it to act as a general acid for the first step of catalysis. This step involves protonating the phenolic oxygen of the tyrosyl leaving group, thus cleaving the phosphate off tyrosine, to form the cysteine-phosphate intermediate. This same aspartate

residue also acts as a general base in the second step of catalysis, which, together with a highly conserved glutamine residue (Gln^{262} in PTP1B), co-ordinates an essential water molecule to promote the hydrolysis of the cysteine-phosphate intermediate.

Domain-swapping experiments between the highly conserved SHP [SH2 (Src homology 2)-domain-containing protein tyrosine phosphatase]-1 and -2 PTPs [8,9] or TCPTP (T-cell PTP) and PTP1B [10], whose catalytic domains exhibit identities of 61 % and 72 % respectively, demonstrate that their catalytic domains contribute directly to substrate specificity. For example, SHP-2 promotes FGF-2 (fibroblast growth factor 2)-induced animal cap elongation in *Xenopus* oocytes, but fails to do so when its PTP domain is replaced with the PTP domain of SHP-1 [9]. Although similar catalytic domain-swapping experiments have shown that PTP1B and TCPTP can exhibit differences in inherent specificity [10], both PTP1B and TCPTP have a preference for tandem phosphorylated substrates that occur in the activation loops of the IR (insulin receptor) and the JAK (Janus kinase) family of PTKs [11]. In PTP1B and TCPTP, a second phosphotyrosine-binding site exists adjacent to the active site, and is defined by Arg^{47}, Asp^{48}, Met^{258} and Gly^{259} (numbering according to PTP1B) [11]. This second site allows for high-affinity binding of bi-phosphorylated substrates by simultaneous engagement of the phosphotyrosines. Gly^{259} is also present in TCPTP, but in other PTPs it is replaced by more bulky residues that sterically prohibit phosphotyrosine binding [2,11]. Although these observations provide insight into the molecular basis for the differences between catalytic domain specificities of PTP1B and TCPTP compared with those of other PTPs, residues that contribute to PTP1B compared with TCPTP specificity differences remain to be defined. In the case of SHP-1 and SHP-2, their respective crystallographic structures point towards specificity differences being attributable to surface-exposed residues [8]. However, for the most part, it is not known which residues contribute to the substrate specificity of individual PTPs. Extensive mutagenesis screens and the acquisition of structures of different PTPs in complex with peptides and protein substrates may ultimately be necessary to provide comprehensive insight into the molecular basis of PTP substrate recognition and specificity.

PRINCIPLES OF SUBSTRATE-TRAPPING

The principles engaged to identify a PTP substrate are based upon the ability of the PTP to bind to its cognate substrate. When the critical active-site cysteine residue within the PTP domain is mutated, typically to a serine residue, the phosphotyrosyl substrate retains the ability to bind to the PTP active site; however, nucleophillic attack on the substrate phosphate cannot occur. PTP C-S (cysteine-to-serine) mutants have served as an important tool that has been utilized extensively for the identification of a number of putative PTP substrates. However, the resolution of the crystal structure for the archetypal PTP, PTP1B, led the way for the generation of novel PTP mutants by the Tonks laboratory that conferred substantially greater PTP–substrate complex affinity than the C-S mutant [12]. Mutagenesis of the critical aspartate residue in the WPD loop of PTP1B to alanine resulted in a dramatic decrease in k_{cat} without affecting the K_m for the peptide substrate. The D-A (aspartate-to-alanine) mutant allowed for the formation of a cysteine-phosphate intermediate thus forming an effective trap for substrates [12]. The D-A substrate-trapping mutant was eagerly anticipated as being the turning point for the identification of PTP substrates. Since then, there has been considerable progress in PTP substrate identification; as will be discussed, some of the identified PTP substrates have provided significant insight into PTP biological function.

Substrate-trapping approaches progressed from the use of the single D-A mutation to the use of double mutations as a means to improve PTP substrate-trapping efficiency. There have been several variations of substrate-trapping mutants that have evolved. Agazie and Hayman [13] generated a double-substrate-trapping mutant wherein the C-S mutant was combined with the D-A mutant in SHP-2. In this instance, the double-substrate-trapping mutant, C-S/D-A, was more effective in substrate-trapping than either mutant alone [13]. In addition to the use of the C-S/D-A substrate-trapping mutant, there have been other substrate-trapping mutant variants. For example, the D-A substrate-trapping mutant in the context of a Q-A (glutamine-to-alanine) mutation (D-A/Q-A) within the PTP active site has been reported to serve as an efficient substrate-trapping PTP mutant for PTP-1B and SHP-2 [14,15]. Here, the highly conserved active-site glutamine residue (Gln^{262} in PTP-1B) stabilizes the water molecule that eventually attacks the cysteine-phosphate intermediate to liberate free phosphate and water. A D-A/Q-A mutation in PTP1B was shown to have a 5-fold increase in substrate-trapping efficiency as compared with either the C-S or the D-A mutant alone [15]. Presumably, the Q-A mutant serves to further cripple any residual phosphatase activity that might exist with the D-A mutant, rendering it more effective in forming a stable enzyme complex. An interesting double-substrate-trapping mutant was also generated with PTPH1. The PTPH1 D-A mutant by itself was found to be incapable of trapping substrates in cells [16]. But when the D-A mutation was combined with a phenylalanine mutation of a tyrosine residue in the catalytic site (PTPH1 D-A/Y-F), which is involved in co-ordinating substrate recognition, substrate-trapping in cells occurred [16]. This tyrosine residue was phosphorylated when PTPH1 D-A was expressed in cells and it was proposed that substrate-trapping was prevented when this tyrosine in PTPH1 was phosphorylated [16].

In general, the approaches that have been applied to identify PTP substrates using the C-S, D-A, C-S/D-A or D-A/Q-A substrate-trapping mutants centre around the ability of these mutated PTPs to interact stably with their cognate substrates. In the vast majority of cases, a stable PTP–substrate complex has been identified through a process of trial and error, and herein lies the major technical bottleneck for identifying PTP substrates. Using commercially available antibodies against proteins that correspond to the apparent molecular mass of the trapped protein, researchers have used intuition and clues about the nature of the signalling pathways in which the PTP is thought to participate as a guide to identifying the trapped substrate. This approach is obviously not very robust, as it relies on an antibody being available against the putative substrate under investigation in addition to the trapped substrate being of a sufficiently high abundance to allow for its detection by immunoblotting. Despite these limitations, this 'best guess' approach has, surprisingly, been very successful. However, it is unlikely that 'hits' from this approach will yield indefinitely, and more sophisticated identification approaches will be required.

A limited number of studies that utilize techniques that are not reliant upon the availability of an antibody for the identification of PTP substrates have been reported. Substrate-trapping approaches for SHP-2 in combination with matrix-assisted laser-desorption ionization spectroscopy have been applied to identify the major vault protein as a SHP-2 substrate [17]. In this approach, lysates from human fibroblasts were used as a source to affinity-purify substrates using the D-A substrate-trapping mutant of SHP-2 [17]. In other applications, there have been attempts to combine the substrate-trapping mutant with modified yeast two-hybrid

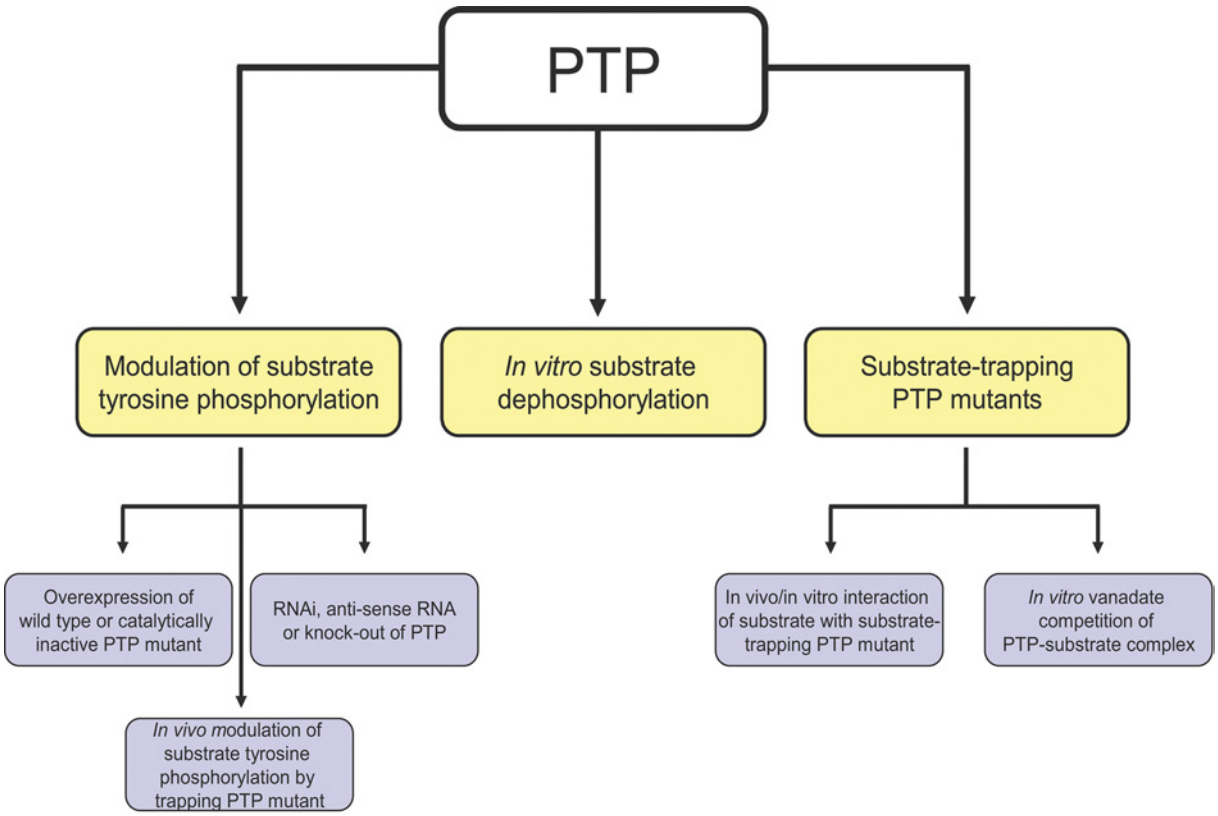

Figure 2 Characterization of PTP substrates

The three proposed criteria for the assignment of a tyrosine-phosphorylated protein as a PTP substrate. To define a tyrosine-phosphorylated protein as a PTP substrate, one should (i) demonstrate interaction of the substrate with the PTP substrate-trapping mutant, (ii) modulate the substrate tyrosine-phosphorylation level in a cellular context, and (iii) dephosphorylate the substrate *in vitro*. A combination of overexpression of the wild-type PTP and substrate-trapping PTP mutant along with underexpression approaches (e.g. RNAi, antisense and knockout cells) can be employed in order to test whether a putative PTP substrate satisfies these criteria (see the text for details).

screens in which cDNA library products are phosphorylated by a constitutively active PTK and the D-A substrate-trapping mutant of the PTP is used as bait. This elegant approach resulted in the identification of the G-protein-coupled receptor kinase-interactor 1 as a putative PTP ζ/β substrate [18]. Although both proteomic and two-hybrid screening approaches have their limitations, in particular the reliance of the PTP–substrate interaction being of sufficiently high affinity and abundance for proteomic detection and/or the use of the appropriate kinase to phosphorylate the products of an expression library, these approaches are attractive because they do not rely upon the availability of antibodies against known molecules for their success. The advancement of future PTP substrate identification will be enhanced greatly with the adoption of these more sophisticated proteomic and molecular genetic screening approaches.

CRITERIA FOR DEFINING PTP SUBSTRATES

A number of putative PTP substrates have been identified. However, the experimental approaches and criteria that have been used to conclude that a particular tyrosine-phosphorylated protein is a PTP substrate vary. In some cases, PTP substrates have been proposed based primarily upon the ability of the PTP to trap its substrate. In other cases, PTP substrates have been defined based upon the effects of either loss-of- or gain-of-function approaches of the PTP that modulates the tyrosine-phosphorylation content of the putative target protein. In other instances, PTP substrates have been suggested based solely on the capacity of the PTP to dephosphorylate a substrate *in vitro*. Each one of these approaches provides suggestive evidence for the identification of a PTP substrate, but they all have inherent limitations. We propose that there should be a minimum experimental standard in order to define a putative PTP substrate as *bona fide*. A similar line of guidance was proposed by Krebs and Beavo [19] for establishing the physiological significance of phosphorylation and dephosphorylation events. The scheme for defining a tyrosine-phosphorylated protein as a PTP substrate is shown in Figure 2 and should attempt to satisfy the following three criteria.

Criterion 1: direct interaction of substrate with the PTP substrate-trapping mutant

Probably one of the most important criteria to be satisfied in defining a PTP substrate is to establish whether the substrate-trapping mutant and putative substrate form a stable enzyme–substrate complex within a cellular context (Figure 2). In this strategy, the substrate-trapping PTP mutant can be overexpressed in the relevant target cell and the PTP–substrate complex is isolated using standard biochemical approaches. The isolated PTP substrate-trapping mutant should form a stable interaction with the tyrosine-phosphorylated endogenous substrate, whereas the wild-type PTP should not interact, or at least if it does, should do so to a lesser extent than the PTP substrate-trapping mutant. The PTP-D-A–substrate complex should also be out-competed by sodium orthovanadate, which acts as a phosphotyrosine mimetic and

covalently modifies the catalytic cysteine residue. The ability of sodium orthovandate to disrupt the PTP-D-A–substrate complex is often overlooked, but it is quite an important verification that the complex between the PTP catalytic domain and the substrate is a direct one. However, in some cases, this approach may be uninformative if, in the context of the full-length PTP, there are additional interactions that are independent of the catalytic domain. In these instances, sodium orthovanadate disruption of the PTP-D-A–substrate complex may be ineffective.

In undertaking substrate-trapping experiments in a cellular context, one can specifically modulate signalling pathways by the addition, for example, of growth factors and thus enhance substrate phosphorylation; this affords the opportunity to link PTP–substrate interactions to specific biological pathways. One of the problems faced with substrate-trapping in cells is that the substrate-trapping mutant must effectively compete with the endogenous PTP. Now that knockouts and RNAi (RNA interference) approaches are commonplace, using these systems in conjunction with the PTP substrate-trapping mutant should eliminate potential complications of the endogenous PTP. Performing substrate-trapping experiments within cells also provides the opportunity to derive spatial information of PTP–substrate complex formation, and hence the subcellular locale of substrate dephosphorylation, using standard immunofluoresence techniques as well as more advanced ones such as fluorescence resonance energy transfer.

In some circumstances, monitoring for an interaction between a PTP substrate-trapping mutant and an endogenous substrate may be limited by the abundance of the substrate or the extent of substrate tyrosine phosphorylation. In these situations, co-expression of the substrate and the PTP substrate-trapping mutant can be performed. Alternatively, one can determine whether a stable interaction can be formed *in vitro* between the purified PTP substrate-trapping mutant and tyrosine-phosphorylated substrate or whether the substrate-trapping mutant can complex with it from a cellular or tissue extract. However, on their own, these types of *in vitro* and overexpression approaches can yield artefacts.

Criterion 2: modulation of cellular substrate tyrosine phosphorylation by the PTP

A second criterion which also should be satisfied toward the goal of unequivocal assignment of a PTP substrate is the demonstration that the endogenous tyrosine-phosphorylation levels of the putative substrate can be modulated by altering the activity of the PTP (Figure 2). As a first step, overexpression approaches in cells can be performed in order to determine whether overexpression of the wild--type PTP correlates with the dephosphorylation of the substrate. However, depending upon how the PTP is regulated, overexpression of the wild-type PTP may not always result in substrate dephosphorylation, as is the case for the SH2 (Src homology 2)-containing PTPs. The substrate-trapping mutant can also be overexpressed and it should enhance substrate tyrosine phosphorylation by forming a stable complex with the substrate and thereby prevent its dephosphorylation by endogenous PTPs. Although, in most cases, the substrate-trapping mutant results in hyper(tyrosine phosphorylation) of the substrate when overexpressed in cells, in some instances, the D-A mutant might not be sufficient to accomplish this, as observed for PTP-H1 [16]. In these situations, either modification of the PTP-D-A mutant or overexpression of alternative PTP mutants that are catalytically inactive, such as the C-S or the R-M (arginine-to-methionine) mutants, should similarly result in the hyper(tyrosine phosphorylation) of the putative substrate.

The type of overexpression approaches discussed should ideally be complemented with loss-of-function approaches to ensure that any result attained is not an artefact of overexpression. This can involve the use of knockout cell lines or cells in which PTP expression has been suppressed by antisense or RNAi approaches. All of these strategies should result in the enhancement of the tyrosine-phosphorylation levels of the substrate, either basally and/or in response to activation of a signalling pathway. Since many substrates are phosphorylated on multiple sites, a possible caveat of these experiments is that the substrate may be discretely dephosphorylated by the PTP at a single site. Therefore the ability to detect an increase in the net levels of substrate tyrosine phosphorylation may be difficult. In this instance, identifying the site of dephosphorylation on the PTP substrate might be necessary. However, the identification of the site of tyrosine dephosphorylation by the PTP is not considered to be an absolute requirement for defining PTP substrates.

Criterion 3: *in vitro* dephosphorylation of substrate by the PTP

The third criterion which should be satisfied is to determine whether the PTP directly dephosphorylates the putative substrate (Figure 2). This is a relatively straightforward approach; however, it is often prone to overinterpretation. In this approach, the purified wild-type PTP, preferably in the context of the full-length molecule, is mixed with the tyrosine-phosphorylated substrate. One can monitor for dephosphorylation of the substrate using anti-phosphotyrosine antibodies or the release of phosphate either colorimetrically or by the release of radiolabelled phosphate. In many cases, the isolated catalytic domain will, to varying extents, dephosphorylate any given substrate. This is due, at least in part, to the often relaxed constraint on PTP domain specificity in the absence of non-catalytic N- and C-termini which serve regulatory roles. For this reason, establishing that a substrate can be dephosphorylated *in vitro* by a PTP is by itself not overly compelling. In addition, in the context of the full-length PTP molecule, the dephosphorylation of a substrate *in vitro* may vary greatly and can depend upon how the PTP is regulated. For example, both SHP-1 and SHP-2 are autoinhibited and become activated when their SH2 domains are bound by a phosphotyrosyl peptide [20]. Therefore *in vitro* dephosphorylation reactions with SHP-1 and SHP-2 are unlikely, in most instances, to be informative unless accompanied by the addition of an activating phosphotyrosyl peptide or a constitutively active mutant form of the PTP [9]. Another caveat of the *in vitro* dephosphorylation approach is that it could lead to an overestimation of the extent to which the PTP can dephosphorylate the substrate. For example, within the cell, there are additional constraints that dictate the subcellular targeting of both the PTP and the substrate. In addition, a PTP may selectively dephosphorylate specific residues within a multi-phosphorylated protein that may not be readily discerned in an *in vitro* context. Such is the case, for example, for PTP1B on the IR [11], TC-PTP on Shc [10], and SHP-2 on Gab-1 [13,21]. Hence, the ability of the PTP to discretely dephosphorylate its substrate to reveal such subtle levels of regulatory dephosphorylation may be difficult to establish *in vitro*. Although often considered to be an important first step in defining a PTP substrate, *in vitro* dephosphorylation experiments should be interpreted cautiously as stand-alone experiments when characterizing PTP substrates.

The successful combination of these criteria should provide compelling evidence for the assignment of a tyrosine-phosphorylated protein as a PTP substrate. We consider the criteria discussed as a complete experimental data set that should allow confident assignment of PTP substrates. In some cases, one might have to

extend these criteria to the identification of specific sites of PTP substrate dephosphorylation as discussed above. Nevertheless, if these criteria are adopted, we anticipate that classifying PTP substrates should become more amenable to broad comparative analysis.

CRITICAL ESSENTIALS AND CAVEATS OF CRITERIA FOR PTP SUBSTRATE IDENTIFICATION

The three criteria discussed in the previous section by themselves are unlikely to provide unequivocal evidence that a tyrosine-phosphorylated protein is a PTP substrate because each of the approaches that comprise these criteria have their own limitations. For example, the *in vitro* dephosphorylation of PTP substrates is sometimes subject to a lack of specificity and so must be complemented with substrate-trapping experiments within cells. The importance of substrate-trapping in cells is critical because it demonstrates that the PTP–substrate complex can coexist in context of the appropriate subcellular compartment in which both endogenous PTP and substrate reside. This is an important point because subcellular localization of PTPs is a critical feature of their regulation and ability to establish signalling specificity. On the other hand, substrate-trapping experiments conducted in cells involve overexpression approaches which too have limitations. Overexpressing PTP-substrate trapping mutants in cells could result in the generation of non-specific interactions. In addition, when overexpressed, the PTP substrate-trapping mutant could become mislocalized, granting it access to substrates that may not otherwise be encountered under physiological levels of expression. For this reason, support for the PTP-substrate-trapping overexpression approaches should be combined with underexpression strategies such as knockout cells or RNAi, or overexpression of a catalytically inactive PTP to demonstrate that the putative PTP substrate becomes hyper-tyrosine-phosphorylated when the expression and/or activity of the PTP is reduced.

As is the case with the molecular elucidation of any given intracellular signalling pathway, issues of cell-type-specificity also exist when identifying potential PTP substrates. In this regard, one can extend the substrate-trapping approaches to different cell types or tissues in order to attain the generality of the substrate for dephosphorylation by the PTP. It is likely that PTPs will dephosphorylate different substrates, on the basis of either the expression of the substrate and/or the phosphotyrosyl status of the substrate in different cell types. Because of the potential that PTP substrates will vary in expression levels, a caveat of the substrate-trapping approach is that it is limited in the detection of lower-abundance PTP substrates. Therefore, in the criteria involving overexpression of the PTP substrate-trapping mutant, demonstration of PTP–substrate complex formation in certain cell types might prove to be difficult if the expression of the PTP substrate is too low. Utilization of *in vitro*, proteomic or molecular genetic approaches as mentioned above should overcome these limitations.

We have discussed the spatial issues of PTP–substrate complex formation in cells; however, the temporal actions of PTP dephosphorylation generates another level of complexity in the identification of PTP substrates. A limitation that needs to be realized with PTP substrate-trapping in cells is that it relies on the generation of stable complexes between the PTP substrate-trapping mutant and its substrate. These complexes might interfere with the propagation of downstream signalling, resulting in the failure of other putative PTP substrates from becoming tyrosine-phosphorylated, hence preventing their detection by the exogenously expressed PTP substrate-trapping mutant. Therefore one should be aware of the consequences that the PTP substrate-trapping mutant might have on the signalling pathway under study and that failure to trap a particular substrate might reflect the inability of the putative substrate to become tyrosine-phosphorylated.

LINKING PTP SUBSTRATES WITH THEIR BIOLOGY

Although the identification of PTP substrates has been challenging, there have been a number of positive developments in the PTP field that clearly demonstrate that these issues can be overcome. However, it is not always clear that an identified substrate will necessarily reveal the molecular basis for the physiological actions of the PTP. This is not to say that the identified substrate is not physiologically relevant. Herein lies the second challenge: the success of defining the molecular basis for PTP signalling will depend upon the ability to identify substrates that provide an explanation of the biological role of the PTP. Fortunately, primarily because of extraordinarily intense research efforts on a select number of PTPs (see below), there are excellent, albeit still limited, examples in which PTP substrates have been identified that make biological sense in terms of explaining the mechanisms of action of a PTP. We will focus our discussion on studies that have identified PTP substrates that explain, or are consistent with, the biology of the PTP in addition to conforming closely to the aforementioned criteria for defining a PTP substrate.

SHP-2 (*PTPN11*)

The non-transmembrane SH2-domain-containing PTP, SHP-2, presents a unique challenge with regards to the identification of its substrates. This is because, based on a wealth of both biochemical and genetic data, SHP-2, unlike other PTPs, which negatively regulate signalling, positively regulates cell signalling [22]. The catalytic activity of SHP-2 is required for the propagation of the ERK (extracellular-signal-regulated kinase) and PI3K (phosphoinositide 3-kinase)/Akt (protein kinase B) pathways downstream of multiple receptor PTKs [22]. The model for SHP-2 signalling is proposed to involve the dephosphorylation of a putative substrate(s) that is negatively regulated by tyrosine phosphorylation. The dephosphorylation of the substrate results in the generation of a positive signal. Although there are a few cases in which SHP-2 acts to negatively regulate cell signalling, much of the substrate identification efforts have focused on uncovering the substrate(s) that control Ras/ERK activation. A number of substrates for SHP-2 have been identified using combinations of the three criteria described and these include Sprouty [23], the signal regulatory protein-α [24,25], PZR (protein zero-related) [26], STAT1 (signal transducer and activator of transcription 1) [27] and STAT5a [28]. However, these putative SHP-2 substrates still require additional characterization.

The earliest evidence for a substrate for SHP-2 that was consistent with its positive role in Ras/ERK signalling came before the advent of substrate-trapping approaches. Work by Klinghoffer and Kazlauskas [29] demonstrated that SHP-2 specifically dephosphorylated the RasGAP (Ras GTPase-activating protein)-binding site at Tyr^{771} and the PI3K-binding site at Tyr^{751} of the PDGFR (platelet-derived growth factor receptor) β *in vitro* [29]. Using mutants of the PDGFR that fail to bind SHP-2, they showed that RasGAP binding was increased, suggesting that SHP-2 may positively signal to Ras/ERK by preventing the association of RasGAP with the PDGFR. Work by Agazie and Hayman [13] supported this theme that SHP-2 might activate Ras/ERK signalling by negatively regulating RasGAP recruitment. Using

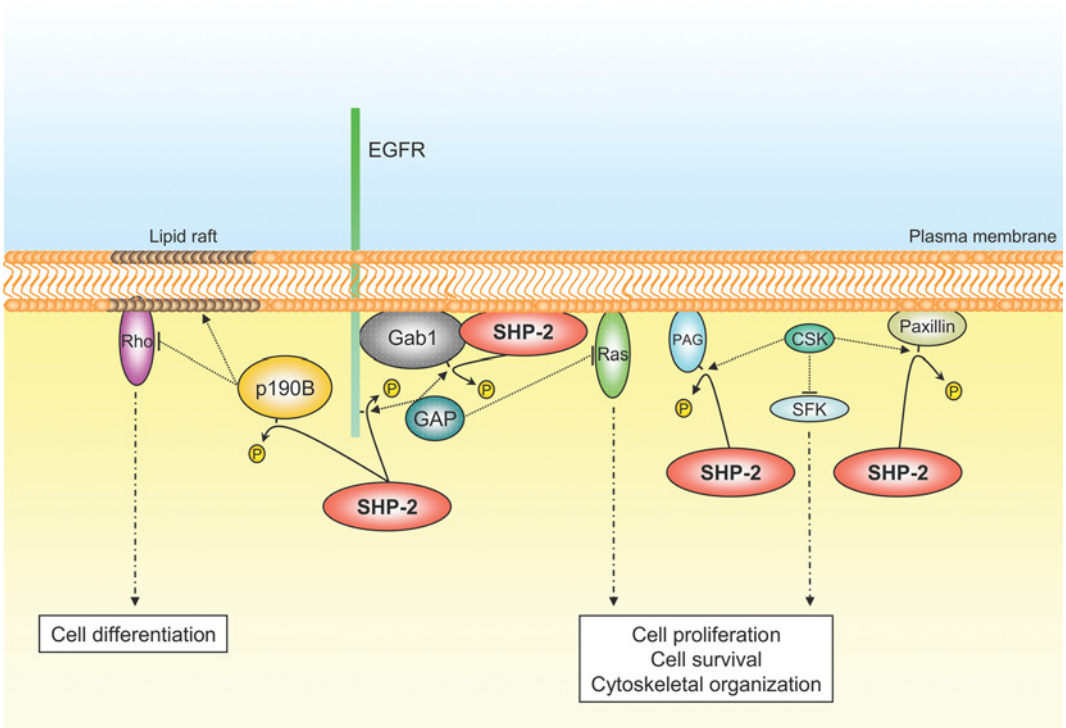

Figure 3 SHP-2 signals positively by inactivating negative regulators of small GTPases and SFKs

SHP-2 dephosphorylates the EGFR (Tyr^{992}) and Gab1 (Grb2-associated binder-1) to prevent the translocation of p120 RasGAP to the EGFR or Gab1 where p120 RasGAP inactivates Ras (broken lines). By interfering with p120 RasGAP localization SHP-2 facilitates the activation of Ras in response to EGF (epidermal growth factor). When phosphorylated, p190B RhoGAP co-localizes with RhoA in lipid rafts to inhibit RhoA activation (broken lines). Dephosphorylation of p190B RhoGAP displaces it from lipid rafts where it is no longer able to inactivate RhoA. In muscle cells, SHP-2 dephosphorylates p190B RhoGAP, thereby promoting RhoA-mediated muscle differentiation. SHP-2 controls localization of the negative regulator of the SFKs, CSK. CSK binds phosphorylated PAG/Cbp and paxillin where it is able to inactivate the SFKs (broken lines). SHP-2 dephosphorylates PAG/Cbp and paxillin causing CSK to dissociate from these complexes preventing it from inhibiting the SFKs. SHP-2 thus promotes SFK activation in the control of cell proliferation, cell survival and cytoskeletal organization.

substrate-trapping approaches (SHP-2-D-A/C-S) they identified the EGFR (epidermal growth factor receptor) as a SHP-2 substrate [13]. They subsequently demonstrated that phospho-Tyr^{992} of the EGFR served as the specific site of dephosphorylation by SHP-2 [30]. Significantly, Tyr^{992} of the EGFR when phosphorylated provides a binding site for RasGAP and, when a catalytically inactive mutant of SHP-2 was overexpressed, RasGAP association to the EGFR was inhibited [30]. Therefore it appears that SHP-2 regulates Ras/ERK activation by negatively regulating RasGAP localization to the plasma membrane (Figure 3). The theme of negative regulation of RasGAPs by SHP-2 has been extended [13,21]. Montagner et al. [21] suggest that Tyr^{317} is a major RasGAP-binding site which is dephosphorylated by SHP-2. However, direct demonstration that SHP-2 dephosphorylates Tyr^{317} on Gab1 was not provided. Nonetheless, this report is supported by *in vivo* observations that show that, in a liver-specific deletion of SHP-2, Gab1 is hypertyrosine-phosphorylated and there is increased RasGAP binding to Gab1 [31]. Collectively, these studies raise a plausible paradigm for positive signalling by SHP-2 *in vivo* through the dephosphorylation of substrates that negatively regulate RasGAP recruitment/activation (Figure 3).

Further mechanistic support for the molecular basis of SHP-2 signalling to Ras/ERK has been proposed. SHP-2 was found to positively signal to the SFKs (Src family kinases) by controlling the localization of the negative regulator of the SFKs, CSK (C-terminal Src kinase) [32]. The mechanisms proposed are through the dephosphorylation of the CSK-binding proteins, PAG (phosphoprotein associated with glycosphingolipid-enriched membrane microdomains)/Cbp (CSK-binding protein) and paxillin, resulting in the release of CSK from the locale where it negatively regulates SFK activation by phosphorylation [32,33]. These reports provide evidence for how SHP-2 signals in a positive manner using primarily overexpression of SHP-2-inactive mutants that resulted in the hyperphosphorylation of PAG/Cbp and paxillin [32,33]. It still remains to be demonstrated whether PAG/Cbp or paxillin are direct SHP-2 substrates within a cellular environment through the use of substrate-trapping. However, this link between SHP-2 and the SFKs provides a plausible explanation for how SHP-2 regulates SFK/ERK signalling (Figure 3).

The substrates of SHP-2 that support a mechanism for signalling to Ras/ERK (EGFR, PDGFR, PAG/Cbp and paxillin) may also be targets of SHP-2 during embryogenesis. Either mutation or deletion of SHP-2 results in early embryonic lethality [34,35]. It is therefore conceivable that loss of Ras/ERK regulation during development is causal to the embryonic lethality in SHP-2 mutant mice. In support of this, Yang et al. [34] demonstrated that SHP-2 is required for trophoblast stem cell survival by promoting the Src/Ras/ERK pathway. Although the identity of the SHP-2 substrate(s) that is critical for initiating Src/Ras/ERK signalling during development remains to be identified, this study convincingly links regulation of the Ras/ERK pathway by SHP-2 with the early lethality observed in SHP-2-deficient mice.

The substrates regulated by SHP-2 are therefore critical for the progression of normal development. This notion has been exemplified by work from Gelb and co-workers, who identified

that mutations in *PTPN11*, which encodes for human SHP-2, is the cause of approx. 50 % of NS (Noonan syndrome) cases [36]. NS is an autosomal dominant disorder occurring in 1:1000 to 1:2500 live births worldwide [37]. NS patients exhibit proportionate short stature, facial dysmorphia, mental retardation, thrombocytopenia and cardiovascular defects [37]. In addition, NS patients are predisposed to developing juvenile myelomonocytic leukaemia [37]. Remarkably, NS mutations map to residues that are involved in constraining SHP-2 in an auto-inhibitory state [36]. Hence, mutation of these residues causes SHP-2 to adopt an 'open conformation', leading to its constitutive activation; presumably this dysregulation causes aberrant dephosphorylation of SHP-2 substrates resulting in the pathogenesis of NS. NS is among an overlapping panel of genetic diseases collectively referred to as neuro-cardio-facial-cutaneous syndromes, all of which have in common aberrant regulation of the Ras/ERK pathway [38]. Significantly, a knock-in mouse model of a NS mutation, D61G, recapitulates many features of the human disease [39]. Moreover, endocardial cushions and embryos derived from the NS knock-in mice exhibit hyperactivation of ERK [39]. It is conceivable that the substrates that have been identified which explain the ability of SHP-2 to stimulate the Ras/ERK axis are good candidates for dephosphorylation by SHP-2 in NS. Further studies to identify these substrates will provide definitive evidence for a more direct mechanistic link between SHP-2 and NS.

Substrates of SHP-2 that have been implicated in disease include FAK (focal adhesion kinase). *CagA* is the virulence gene of *Helicobacter pylori* and infection with *cagA*-positive *H. pylori* results in the tyrosine phosphorylation of CagA and its association with SHP-2 [40]. Patients infected with *cagA*-positive *H. pylori* have increased risk of gastric disease and also CagA–SHP-2 complexes have been detected in the gastric mucosa of these patients [40]. Work from Tsutsumi et al. [41] studying the mechanisms of *H. pylori* CagA-mediated signalling demonstrate that FAK is a specific SHP-2 substrate in response to CagA infection of gastric epithelial cells. Infection of cells with *CagA*-positive *H. pylori* results in a dramatic change in the cytoskeleton causing cells to adopt a morphology that is referred to as the 'humming bird' phenotype [40,42]. Dephosphorylation of FAK by SHP-2 is proposed to cause this humming bird phenotype which impairs cell adhesion and increases cell motility of infected gastric epithelia. These effects are believed to increase the propensity of infected mucosa epithelia to succumb to gastric diseases. Whether there is decreased FAK tyrosine phosphorylation in the gastric mucosa of patients infected with *cagA*-positive *H. pylori* remains to be determined. Nevertheless, these results provide an important basis for defining how SHP-2 substrate(s) are involved in disease.

In addition to signalling to Ras, SHP-2 also appears to regulate other small GTPases. Using the SHP-2-D-A/Q-A substrate-trapping mutant, Kontaridis et al. [14] showed that p190B RhoGAP is a SHP-2 substrate. Overexpression of a catalytically inactive mutant of SHP-2 in muscle cells increased p190B RhoGAP tyrosine phosphorylation and vanadate disrupted the association between SHP-2-D-A/Q-A substrate-trapping mutant and tyrosine-phosphorylated p190B RhoGAP [14]. Collectively, these results provided strong evidence that p190B RhoGAP is a *bona fide* SHP-2 substrate. It had been long realized that RhoA is essential for myogenesis, but how RhoA becomes activated in muscle cells was unknown. Dephosphorylation of p190B RhoGAP results in its dissociation from lipid rafts, where RhoA resides, resulting in increased RhoA activity [43]. In muscle cells, SHP-2 dephosphorylates p190B RhoGAP, resulting in the activation of RhoA and subsequently induction of genes required for muscle differentiation [14] (Figure 3). Recently, tissue-specific ablation of SHP-2 in skeletal muscle has been shown to impair skeletal muscle growth through a pathway involving activation of NFAT (nuclear factor of activated T-cells) which is required to promote muscle growth [44]. The RhoA (A. M. Bennett, unpublished work) and SFK/Ras/ERK [44] pathways are required for NFAT activation in muscle cells. Hence, the identification of p190B RhoGAP as a SHP-2 substrate that promotes RhoA activation of NFAT is consistent with a role for SHP-2 in skeletal muscle growth.

PTP-1B (*PTPN1*)

Numerous substrates have been identified for the prototypic PTP1B. Those substrates largely conforming to the criteria discussed above, where substrate-trapping and/or overexpression approaches have been combined with validating underexpression or knockdown strategies, include receptor PTKs such as EGFR [12,45,46], PDGFR [45–47], CSF-1 (colony-stimulating factor 1) receptor [48], IR [49–54] and IGF-1 (insulin-like growth factor-1) receptor [50,55], as well as cytoplasmic PTKs such as c-Src [56–58] and JAK2 [59–62] and the adaptor protein, $p62^{Dok}$ [63]. Other putative substrates identified by overexpression and/or substrate-trapping approaches include Tyk2 (tyrosine kinase 2) [59], BCR (B-cell receptor)-Abl [64], STAT5a/b [65] and $p130^{cas}$ [63,66–68]. In addition, the IRS (IR substrate) proteins 1 and 2 are hyperphosphorylated in cells and tissues from PTP1B-deficient mice [52], and PTP1B can dephosphorylate IRS-1 *in vitro* [69]. However, it remains unclear whether PTP1B acts directly on IRS proteins *in vivo*. With the exception of the IR and JAK2, the validation and assessment of physiological PTP1B substrates has been limited to an assessment of the tyrosine-phosphorylation status of proteins following activation of signalling pathways in MEFs (mouse embryo fibroblasts) isolated from PTP1B-deficient mice. As such, although many of the substrates identified above may be *bona fide*, the biological context under which they may be regulated by PTP1B remains to be resolved.

Probably the best understood, and most extensively studied PTP1B substrates, are the IR and JAK2 (Figure 4). A large body of *in vitro* and *in vivo* studies has served to establish PTP1B as an integral regulator of metabolic processes having a principal role in the maintenance of glucose homoeostasis and body mass by antagonizing IR and JAK2 autophosphorylation and activation [49–54,59–61,70,71]. PTP1B-deficient mice exhibit enhanced insulin-sensitivity attributable to increased IR activity and downstream IRS phosphorylation in the liver and skeletal muscle [52,54,60,70]. In addition, PTP1B-deficient mice exhibit resistance to diet-induced obesity [52,70] owing to increased hypothalamic leptin-induced JAK2 signalling [60,61,71] – leptin being the key adipokine that is responsible for limiting food intake and increasing energy expenditure in mammals. Consistent with its role in both IR and leptin-induced JAK2 tyrosine phosphorylation, PTP1B deficiency can alleviate diabetes and decrease weight in leptin-deficient obese mice [60] and can improve glucose tolerance and delay the onset of diabetes in IRS-2-knockout mice [72]. In contrast, transgenic overexpression of PTP1B in muscle causes insulin resistance by suppressing IR signalling [53].

PTP1B's role in IR inactivation in liver and muscle and the suppression of leptin-induced JAK2 activation in the hypothalamus has led to a considerable amount of attention being paid to PTP1B as a target for the development of therapeutics against Type 2 diabetes and obesity. Notably, preclinical studies utilizing antisense oligonucleotides that suppress PTP1B expression in mouse and rat models of insulin resistance have demonstrated that inhibition of PTP1B expression enhances insulin-sensitivity and

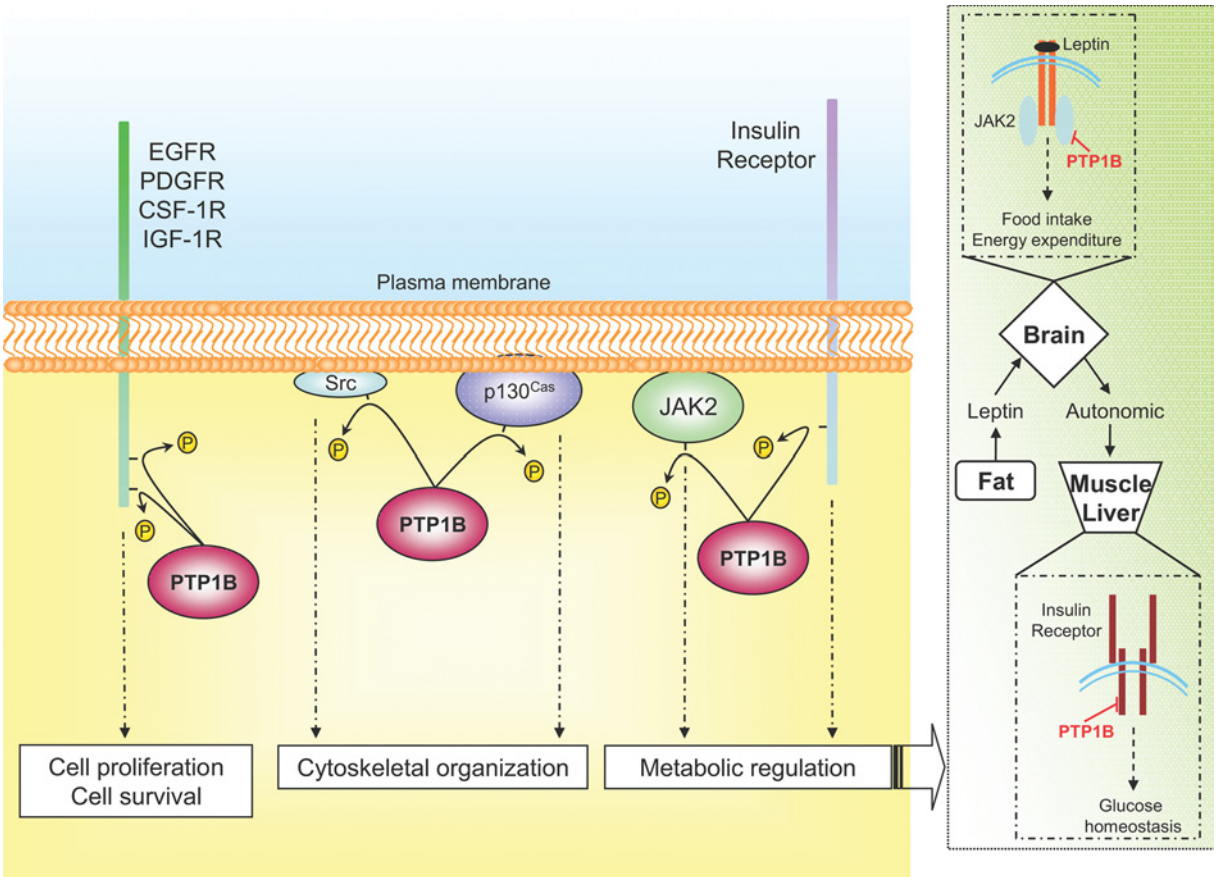

Figure 4 Physiological roles of PTP1B substrates

A number of receptor tyrosine kinases are dephosphorylated by PTP1B, including EGFR, PDGFR, CSF-1 receptor (CSF-1R) and IGF-1 receptor (IGF-1R). Dephosphorylation of these receptors by PTP1B antagonizes receptor functions such as cell proliferation and survival. c-Src and $p130^{Cas}$ serve as PTP1B substrates in the control of cytoskeletal organization. In the case of c-Src, dephosphorylation at Tyr^{527} by PTP1B activates c-Src. PTP1B dephosphorylates the IR (Tyr^{1162}/Tyr^{1163}) and JAK2 (Tyr^{1007}/Tyr^{1008}). Right-hand panel: IR and JAK2 are involved in metabolic homoeostasis and PTP1B functions in the brain to dephosphorylate JAK2 to limit leptin signalling in the control of food intake and energy expenditure. In peripheral tissues, PTP1B dephosphorylates the IR to inhibit glucose uptake in skeletal muscle and liver.

normalizes blood glucose [73–75]. Antisense oligonucleotides targeting human PTP1B are in Phase II clinical trials, whereas PTP1B-inhibitory drugs targeting either the PTP1B active site (reviewed in [76]) or novel allosteric regulatory sites [77] are under development. PTP1B therefore serves as an excellent example whereby linking PTPs and their substrates to biology may afford the opportunity for the development of novel therapeutics for the treatment of human diseases.

PTP1B might also act on additional substrates that may be important in pathological processes. For example, several studies relying on the overexpression of wild-type and mutant forms of PTP1B and the characterization of PTP1B-deficient MEFs have implicated PTP1B in the regulation of integrin signalling, cell spreading and migration via the regulation of c-Src and possibly $p130^{cas}$ phosphorylation [56–58,66,67,78] (Figure 4). In particular, PTP1B may be required for c-Src activation by dephosphorylating the inhibitory CSK tyrosine phosphorylation site (Tyr^{529}; avian Tyr^{527}) on c-Src [56–58,78]. A recent study has provided insight into at least one context for which c-Src regulation by PTP1B may be pertinent *in vivo*. Using platelets isolated from PTP1B-deficient mice, Arias-Salgado et al. [78] have demonstrated that PTP1B is necessary for integrin αIIbβ3-induced c-Src activation and spreading on fibrinogen *in vitro* and for associated calcium signalling and thrombus formation *in vivo*. These findings, although interesting, await further studies to establish their physiological relevance, since PTP1B-deficient mice do not appear to exhibit signs of bleeding diathesis. Although the principal role of PTP1B *in vivo* is to regulate IR activation, glucose homoeostasis and leptin-induced JAK2 signalling (Figure 4), it is becoming increasingly apparent that at least some of the validated PTP1B substrates may play roles in the pathogenesis of certain diseases.

Recent studies also indicate that PTP1B plays an important role in immune cell development and function [48,79]. In particular, Heinonen et al. [48] have demonstrated using substrate-trapping approaches and by assessing responses in PTP1B-deficient mice that PTP1B is capable of dephosphorylating the CSF-1 receptor to control monocyte development. In addition, LPS and IFN (interferon) γ-induced responses are elevated in PTP1B-deficient macrophages *in vitro*, and these mice are hypersensitive to LPS-induced endotoxaemia [48]. Other PTP1B substrates that may be important under pathophysiological conditions include the HGF (hepatocyte growth factor)/Met receptor. Sangwan et al. [80] have reported that PTP1B-deficient mice are protected from Fas-induced hepatic failure. Although their results are consistent with the protection from liver failure correlating with enhanced HGF/Met activity, demonstration that the HGF/Met receptor is a direct PTP1B substrate remains to be established.

TCPTP (*PTPN2*)

Several substrates have been identified for the ubiquitously expressed TCPTP. Those conforming to the criteria described above include the IR [51,81–83], the CSF-1 receptor [84], EGFR [10,85–87], SFKs [88], JAK1 [89] and STAT1 [90]. Other proteins, including the adaptor protein $p52^{Shc}$ [10] and JAK3 [89], as well as STAT3 [91] and STAT5 [92], have also been reported to be TCPTP substrates based on overexpression and substrate-trapping approaches, but await validation by loss-of-function approaches. Site-selective increases in PDGFR tyrosine phosphorylation have also been reported in TCPTP-deficient MEFs [93], but substrate-trapping approaches to demonstrate that TCPTP acts directly on PDGFR have not been conducted.

Unlike PTP1B-deficient mice, where the predominant phenotype can be attributed to elevated IR activation in liver and muscle and leptin-induced JAK2 activation in the hypothalamus [52,60,61,70], the phenotype of TCPTP-deficient mice is much more complex [94]. TCPTP-deficient mice are viable, but have defects in the haemopoietic compartment that result in splenomegaly, lymphadenopathy and thymic atrophy by 2 weeks of age. By 3–5 weeks of age, TCPTP-deficient mice develop a hunched posture and diarrhoea, succumbing to severe anaemia [94]. TCPTP-deficient mice have widespread lymphocytic infiltrates in non-lymphoid tissues correlating with increased IFN-γ, TNF-α (tumour necrosis factor α), interleukin-12 and nitric oxide production [48]. In addition, macrophages from TCPTP-deficient mice are hypersensitive to LPS-induced endotoxic shock [48]. These observations are consistent with TCPTP being an integral component of haemopoietic development and suggest that it serves as a negative regulator of inflammatory responses. Although hyperphosphorylation of JAKs, STATs, SFKs and the receptor for CSF-1 may contribute to the inflammatory/haemopoietic defects of TCPTP-deficient mice, the relative contribution of these substrates to the overall phenotype is unclear. Moreover, the identity of the substrate(s) that are causal to the lethality of TCPTP-deficient mice remains to be determined.

Overexpression, substrate-trapping, knockdown and/or gene knockout approaches have established that both IR and EGFR are *bona fide* TCPTP substrates [10,51,81–83,85–87]. In the case of the IR, Galic et al. [81] have reported that TCPTP can act in concert with PTP1B to control the intensity and duration of IR activation and differentially modulate the phosphorylation of specific IR sites. In contrast, for EGFR, PTP1B and TCPTP may act in different cell types and tissues. Whereas EGFR phosphorylation is elevated in PTP1B-deficient MEFs [45], it is not altered in TCPTP-deficient MEFs (T. Tiganis, unpublished work). At this stage, the context in which TCPTP may regulate these receptor PTKs *in vivo* is unknown.

PEP [PEST (Pro-Glu-Ser-Thr) domain phosphatase]/LYP (lymphoid phosphatase) (*PTPN22*) and SHP-1 (*PTPN6*)

The cytoplasmic PTPs, PEP and SHP-1, are expressed primarily in cells of haemopoietic origin, although SHP-1 expression has also been reported in cells of non-haemopoietic origin. PEP has a large non-catalytic C-terminus containing proline-rich sequences, whereas SHP-1 has a non-catalytic N-terminus containing two SH2 domains. Their respective non-catalytic C- and N-termini mediate interactions with substrates and/or adaptor/targeting proteins [20,95]. In the case of SHP-1, its N-terminus regulates activity in a manner similar to that of SHP-2 [96]. Both PTPs have been implicated in the regulation of lymphocyte signalling, in particular that of the TCR (T-cell receptor) (reviewed in [97,98]); SHP-1 also has been implicated in BCR signalling [99].

The naturally occurring inactivating mutation in murine SHP-1 leads to the motheaten phenotype which manifests as a hyperproliferative disorder in B- and T-cells, impaired natural killer cell function and differentiation and alterations in erythropoiesis [99,100]. The motheaten phenotype clearly establishes SHP-1 as a negative regulator of haemopoietic signalling. In support of this idea, a number of signalling molecules which positively regulate haemopoietic function have been suggested to be putative SHP-1 substrates. These include Lck [101], Syk [102], CSF-1 receptor [103], BIT (brain immunoglobulin-like molecule with tyrosine-based activation motifs) [103], PIR-B (paired immunoglobulin-like receptor B) [103], B-cell linker protein [104], SLP-76 (SH2 domain-containing leucocyte protein of 76 kDa) [105], CD72 [106], Vav [107], p85 [108], $p62^{Dok}$ [109], carcinoembryonic-antigen-related cell adhesion molecule-1 [110] and IR [110]. The assignment of these molecules as putative SHP-1 substrates is certainly consistent with a negative role for SHP-1 in both haemopoietic and non-haemopoietic signalling. However, it has yet to be demonstrated whether all of these targets are direct SHP-1 substrates. Convincing data has been provided demonstrating that BIT [103], PIR-B [103] and CD72 [106] are direct SHP-1 substrates; those studies employed a combination of SHP-1 substrate-trapping, overexpression and *in vitro* dephosphorylation approaches to support this conclusion.

Following TCR engagement, the receptor PTP, CD45, dephosphorylates and activates Lck and Fyn, which then phosphorylate the TCR to recruit and activate ZAP-70 (ζ-chain-associated protein kinase of 70 kDa), culminating in the propagation of various signalling pathways (reviewed in [97]). Through the use of overexpression and substrate-trapping approaches, a number of proteins integral to TCR signalling, including Lck, Fyn, ZAP-70, TCRζ and CD3ε, as well as Vav, have been implicated as substrates for the tyrosine phosphatase PEP [111,112]. However, with the exception of Lck and ZAP-70, it is not clear whether PEP acts directly on the other substrates *in vivo*. PEP-deficient mice are normal, but develop splenomegaly and lymphadenopathy as they age, owing to a specific expansion of their effector/memory T-cell pool [113]. The effector/memory T-cell expansion in PEP-deficient mice is due to elevated Lck and ZAP-70 activation, resulting in increased proliferation (Figure 5). Despite the expression of PEP in peripheral T- and B-cells, Lck-mediated T- and B-cell responses are unaltered in PEP-deficient mice [113]. Thus the restricted phenotype of the PEP-deficient mice underscores the importance of assessing the *in vivo* context in which PTPs recognize their substrates.

Recently, an SNP (single-nucleotide polymorphism) identified in *PTPN22* encoding the human orthologue of PEP, LYP, was linked to the development of various autoimmune diseases, including Type 1 diabetes, rheumatoid arthritis, systemic lupus erythaematosus and Graves disease [114]. The autoimmunity-predisposing SNP is a misssense C-T mutation at position 1858 which changes amino acid 620 of LYP in the C-terminal non-catalytic region from arginine to tryptophan [114]. This change is a gain-of-function mutation allowing for the dephosphorylation and inactivation of the LYP substrate Lck in T-cells [115]. It is proposed that the *PTPN22* mutation may predispose individuals to autoimmune diseases by suppressing TCR signalling and preventing the negative selection of autoreactive thymocytes or by suppressing the activity of regulatory T-cells [115]. However, this requires formal demonstration through the generation of knock-in mice harbouring the *PTPN22* mutation.

PTP-PEST (*PTPN12*)

PTP-PEST is ubiquitously expressed and it has a large non-catalytic C-terminus containing proline-rich sequences that

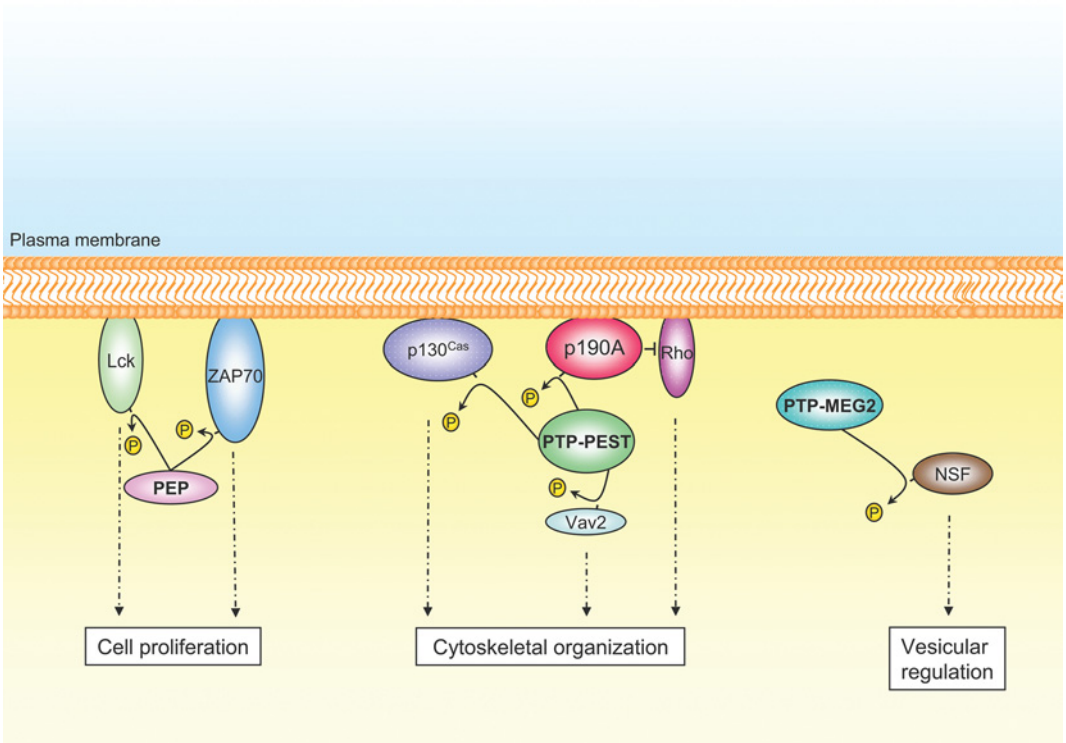

Figure 5 Conforming to the criteria of substrate identification

Substrates of PEP, PTP-PEST and PTP-MEG2 are shown. Proliferation of effector/memory T-cells is negatively regulated by PEP, which dephosphorylates and inactivates Lck and ZAP-70. PTP-PEST regulates cytoskeletal organization by dephosphorylating p130^{Cas}, p190A RhoGAP and Vav2. PTP-MEG2 dephosphorylates NSF (Tyr^{83}) to regulate secretory vesicle fusion.

mediate interactions with substrates/adaptors [95,116–118]. Numerous PTP-PEST substrates have been identified and collectively they point towards PTP-PEST having a primary role in cytoskeletal rearrangement to impact on processes such as cell migration, cell spreading and cell division [66,118–122]. Consistent with this fundamental biological role, and in contrast with the phenotype of PEP-deficient mice [113], PTP-PEST-deficient mice are embryonic lethal [118]. PTP-PEST substrates that conform to the criteria discussed above include the focal adhesion protein p130^{cas} [66,119], the PTK c-Abl [121], Vav2 and p190RhoA GAP [122] (Figure 5). Other putative substrates identified by substrate-trapping or by direct association with PTP-PEST include Pyk2 [123], WASP (Wiskott–Aldrich syndrome protein), which promotes actin nucleation and the WASP-interacting protein, PSTPIP (proline/serine/threonine-phosphatase-interacting protein). In addition to serving as a putative substrate, PSTPIP may also have a targeting role by interacting with the proline-rich PTP-PEST C-terminus, and targeting it for the dephosphorylation of c-Abl, WASP [120,121,124–126] and the paxillin kinase linker [127].

p130^{cas} is a major physiological PTP-PEST substrate, since overexpression of PTP-PEST suppresses p130^{cas} phosphorylation and PTP-PEST substrate-trapping mutants form efficient complexes *in vitro* and *in vivo* [66,116]. These complexes are dependent primarily on direct recognition of the tyrosine-phosphorylated p130^{cas} by the PTP-PEST catalytic domains but also on an interaction between the PTP-PEST proline-rich sequence and the p130^{cas} SH3 domain [66,116]. Importantly, p130^{cas} is hyperphosphorylated in PTP-PEST-deficient MEFs and this correlates with enhanced migration [120], whereas PTP-PEST overexpression suppresses motility [119]. Recently, studies by Sastry et al. [122] have suggested that the hyperphosphorylation of p130^{cas} may not be the primary cause for the enhanced migration of PTP-PEST-deficient fibroblasts. Instead, they suggest that PTP-PEST may suppress cell motility by dephosphorylating and inactivating Rac, the GEF (guanine-nucleotide-exchange factor) Vav2, and p190 RhoA GAP [122]. Dissecting the relative contributions of p130^{cas}, Vav2 and p190 RhoA GAP as PTP-PEST substrates in the control of cell motility and other cytoskeletal-dependent processes is an important challenge that remains to be resolved.

PTP-MEG2 (*PTPN9*)

PTP-MEG2 contains a unique 250-amino-acid non-catalytic N-terminus that has homology with cellular retinaldehyde-binding protein and Sec14p, a yeast protein with phosphatidylinositol-transfer activity. PTP-MEG2 is expressed in various cell types and is targeted to the secretory vesicles and granules of neutrophils and lymphocytes [128,129]. The Sec14p domain interacts with phosphoinositides and phosphatidylserine and is responsible for targeting the enzyme to secretory vesicles [130–132]. Overexpression of PTP-MEG2 in mast cells and Jurkat T-cells causes vesicle fusion, resulting in a dramatic enlargement of vesicles that is dependent on PtdIns(3,4,5)P_3 binding by the Sec14p domain as well as PTP-MEG2 phosphatase activity [130,133]. The first identified substrate for PTP-MEG2 was the vesicle fusion protein NSF (*N*-ethylmaleimide-sensitive factor) [134]. NSF is involved in the disassembly of *cis*-SNARE (NSF-attachment protein receptor) protein complexes, a critical step in the formation of *trans*-SNARE complexes at the site of contact

between vesicle and target membrane compartments. Huynh et al. [134] demonstrated that NSF can be phosphorylated on Tyr^{83} and that this phosphorylation increased NSF ATPase activity. Expression of a NSF-Y83F mutant in Jurkat T-cells resulted in more numerous and larger vesicles [134]. Importantly, PTP-MEG2-D-A substrate-trapping mutant formed a stable complex with tyrosine-phosphorylated NSF in cells [134]. Thus PTP-MEG may be a physiological regulator of secretory vesicle fusion through the control of NSF (Figure 5). Consistent with PTP-MEG2 being integral to vesicle fusion, T-cells isolated from $RAG2^{-/-}$ mice transplanted with PTP-MEG2-deficient embryonic liver-derived progenitor cells are almost devoid of mature secretory vesicles and are defective in interleukin-2 production and therefore T-cell activation [135]. However, it should be noted that NSF hyperphosphorylation has not been shown in PTP-MEG2-deficient T-cells, or, for example, after PTP-MEG2 knockdown by RNAi. As such, the possibility that additional substrates may contribute to the role of PTP-MEG2 in vesicle fusion *in vivo* cannot be excluded.

STEP (striatal-enriched PTP) (*PTPN5*) and HePTP (*PTPN7*)

STEP is preferentially expressed in the neurons of the basal ganglia, hippocampus and cortex [136]. There are two STEP isoforms generated from alternative splicing that migrate with relative molecular masses of 46 and 61 kDa which are named $STEP_{46}$ and $STEP_{61}$, respectively [136]. Both $STEP_{46}$ and $STEP_{61}$ contain a KIM (kinase-interacting motif) which constitutes the binding site for members of the MAPK family [136,137]. KIM domains are not unique to STEP, as HePTP and PTP-SL also contain KIM domains that bind the MAPKs [137]. $STEP_{46}$ and $STEP_{61}$ have been shown to be regulators of ERK in neurons [138]. There is good evidence provided by a substrate-trapping STEP C-S mutant, *in vitro* dephosphorylation of phosphorylated ERK with $STEP_{46}$, and overexpression of loss-of-function mutants of STEP to support the conclusion that ERK is a specific STEP substrate [137,138]. This conclusion is bolstered further by the recent observation that STEP-deficient mice exhibit enhanced ERK activity (P. Lombroso, personal communication). The ability of STEP to modulate ERK activity in neurons is proposed to be important for the mediation of long-term memory such as fear conditioning. HePTP also dephosphorylates ERK; however, unlike STEP, HePTP is predominately expressed in haemopoietic cells. Overexpression of a catalytically inactive mutant of HePTP has been shown to negatively regulate ERK activation and ERK-mediated events such as regulation of AP-1 (activator protein 1) and AP-1 co-operation to activate NFAT [139]. However, the physiological role of HePTP remains to be elucidated, since mice lacking HePTP do not exhibit differences in haemopoietic development or lymphocyte activation, even though ERK is hyperactivated in response to PMA and TCR engagement [140].

CONCLUDING REMARKS

In the last decade, tremendous progress has been made in the identification of PTP substrates. However, a careful assessment of these studies in which PTP substrates have been reported reveals a varied set of experimental approaches that have been employed in order to define whether a tyrosine-phosphorylated protein exhibits the properties of a PTP substrate. As the PTP field matures, and as the list of putative PTP substrates increases, a need to establish criteria by which PTP substrates can be defined in a consistent manner is warranted. Here we propose three criteria as an experimental standard for the assignment of a *bona fide* PTP substrate: (i) direct interaction of the substrate with the PTP substrate-trapping mutant; (ii) modulation of the cellular substrate tyrosine phosphorylation by the PTP; and (iii) *in vitro* dephosphorylation of the substrate by the PTP.

It is of course unreasonable to expect that in all cases the PTP substrate will satisfy all of these criteria. In situations where there are exceptions, a combination of at least two of the three criteria should be satisfied. This strategy, if implemented, should be of great benefit to those in and outside the PTP field, because classifying tyrosine-phosphorylated proteins as PTP substrates in a standard manner should result in the generation of a 'trusted' data set of defined PTP substrates. With the application of more advanced proteomic approaches, the identification of PTP substrates will undoubtedly increase and hence the significance of a clear definition of what constitutes a PTP substrate will become more important. The fidelity of the 'PTP substrate proteome' should make bioinformatic and pathway-mapping analyses of PTPs and their substrates a powerful resource for future research. Such developments will hopefully galvanize further efforts towards defining PTP function which could ultimately lead to the development of novel therapeutics for the treatment of human diseases such as diabetes, obesity, cancer and neurological disorders.

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