Comparative study of protein tyrosine phosphatase- ϵ isoforms: membrane localization confers specificity in cellular signalling

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To study the influence of subcellular localization as a determinant of signal transduction specificity, we assessed the effects of wild-type transmembrane and cytoplasmic protein tyrosine phosphatase (PTP) ϵ on tyrosine kinase signalling in baby hamster kidney (BHK) cells overexpressing the insulin receptor (BHK-IR). The efficiency by which differently localized PTP ϵ and PTP α variants attenuated insulin-induced cell rounding and detachment was determined in a functional clonal-selection assay and in stable cell lines. Compared with the corresponding receptor-type PTPs, the cytoplasmic PTPs (cytPTPs) were considerably less efficient in generating insulin-resistant clones, and exceptionally high compensatory expression levels were required to counteract phosphotyrosine-based signal transduction. Targeting of cytPTP ϵ to the plasma membrane via the Lck-tyrosine kinase dual acylation motif restored high rescue efficiency and abolished

the need for high cytPTP ϵ levels. Consistent with these results, expression levels and subcellular localization of PTP ϵ were also found to determine the phosphorylation level of cellular proteins including focal adhesion kinase (FAK). Furthermore, PTP ϵ stabilized binding of phosphorylated FAK to Src, suggesting this complex as a possible mediator of the PTP ϵ inhibitory response to insulin-induced cell rounding and detachment in BHK-IR cells. Taken together, the present localization–function study indicates that transcriptional control of the subcellular localization of PTP ϵ may provide a molecular mechanism that determines PTP ϵ substrate selectivity and isoform-specific function.

Key words: cell adhesion, dephosphorylation, insulin receptor, Src protein tyrosine kinase, PTP.

INTRODUCTION

Phosphorylation of tyrosine residues constitutes a reversible covalent modification of proteins that acts to regulate essential cellular processes, such as cell proliferation, migration, differentiation and apoptosis (reviewed in [1,2]). Since aberrant protein tyrosine phosphorylation has been linked to both oncogenic and metabolic disease states, tight and specific control of these processes is essential.

This specificity, which ensures controlled activation of phosphotyrosine-based signalling, is achieved at several levels. First, site-specific protein phosphorylation and dephosphorylation is conveyed by the inherent substrate specificity that resides within the catalytic domains of protein tyrosine kinases (PTKs) [3] and protein tyrosine phosphatases (PTPs) [4,5]. Secondly, tyrosine phosphorylation can induce conformational changes in the tertiary structure that directly control the catalytic activity of PTKs and PTPs {e.g. Src [6] or SHP2 (GenBank[®] accession number D13540) [7]}. Thirdly, phosphorylation of specific tyrosine residues in cellular substrates imparts specificity in protein–protein interactions by acting as recognition motifs for phosphotyrosine-binding modules, such as Src homology 2 (SH2) and phosphotyrosine binding domains ('PTB domains') [8]. Fourthly, the duration and amplitude of cell signal reactions adds a further level of control [9,10]. Finally compartmentalization of signalling molecules conveys specificity to cell signalling by influencing accessibility to substrates and associated proteins [11,12].

Since isolated catalytic PTP domains seem to possess relatively low substrate selectivity towards synthetic phosphopeptides *in vitro*, it has been suggested that the correct localization of PTPs in cells is a major determinant of substrate specificity [11]. Indeed, for non-transmembrane PTPs, confinement to a specific subcellular localization is determined by highly divergent targeting sequences, that include membrane-association domains, nuclear-localization domains, SH2 domains and cytoskeletal-association domains [11]. As an example, PTP1B localizes to the endoplasmic reticulum (ER) via its C-terminal amino acid sequence [13]. Similarly, the 48 kDa TC-PTP isoform (GenBank[®] accession number M25393) associates with the ER, whereas an alternatively spliced 45 kDa form is targeted to the nucleus [14].

Although the subcellular localization and thereby the signalling specificity of PTP1B is further regulated post-translationally by proteolytic cleavage [15], the compartmentalization of other PTP domains is determined at the post-transcriptional or transcriptional level. Thus a cytosolic splice variant of receptor-type PTP (RPTP) Φ mRNA has been identified [16], and for PTP ϵ the alternative usage of isoform-specific 5' exons and promoters generates both cytoplasmic and transmembrane enzymes, which can be further proteolytically processed *in vivo* [17–20].

To gain insight into the role of subcellular localization as a determinant of signalling specificity we compared the effect of the cytoplasmic and transmembrane forms of PTP ϵ on phosphotyrosine-based signal transduction. A previously developed insulin-dependent selection assay in baby hamster kidney (BHK)

Abbreviations used: BHK, baby hamster kidney; BHK-IR, BHK cells overexpressing the insulin receptor; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FAK, focal adhesion kinase; Grb2, growth-factor-receptor-bound protein 2; HRP, horseradish peroxidase; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; cytPTP, cytoplasmic PTP; RPTP, receptor-type PTP; SH2, Src homology 2.

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cells overexpressing the insulin receptor (BHK-IR) was chosen as the experimental model, since it allows a quantitative assessment of localization–function relationships [21]. In these cells, insulin receptor activation leads to cell rounding and cell detachment, whereas expression of molecules such as PTPs may attenuate these effects to varying degrees depending on their ability to counteract the activated signalling pathway(s). By targeting the cytoplasmic PTP (cytPTP) ϵ to the plasma membrane, using the Lck-dual acylation motif [22], we demonstrate that the extracellular domain of RPTP ϵ is dispensable, whereas membrane localization is critical for opposing ligand-induced PTK signalling. The pronounced difference in efficiency by which cytPTP ϵ and RPTP ϵ counteract phosphotyrosine-based signalling in BHK-IR cells indicates that transcriptional control of the subcellular localization of PTP ϵ provides a molecular mechanism that may regulate PTP ϵ substrate specificity and biological function *in vivo*.

MATERIALS AND METHODS

Antibodies

For immunoblotting, the primary mouse monoclonal antibodies used were: anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY, U.S.A.) and anti-(insulin receptor) antibody CT-21 (kindly provided by Kenneth Siddle, University of Cambridge, U.K.). Polyclonal antiserum recognizing both PTP ϵ and PTP α was generated in rabbits using a keyhole-limpet-haemocyanin-coupled C-terminal peptide of PTP α [23]. Primary antibodies used for immunofluorescence studies were: rabbit anti-PTP ϵ serum (V5012; kindly provided by Peter Kastrup, Dako A/S, Copenhagen, Denmark), and PTP α -selective mouse monoclonal 2F23A5 antibody directed against an epitope within the N-terminal catalytic PTP domain (Novo

Nordisk). For immunoprecipitation, agarose-conjugated anti-(focal adhesion kinase) (FAK) polyclonal antibody (Upstate Biotechnology) and agarose-conjugated anti-Src monoclonal antibody (Calbiochem–Novabiochem, San Diego, CA, U.S.A.) were used. Immunoprecipitates were analysed with either anti-FAK monoclonal antibody (Transduction Laboratories, San Diego, CA, U.S.A.), anti-Src polyclonal antibody (N-16; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or horseradish peroxidase (HRP)-conjugated phosphotyrosine antibody 4G10 (Upstate Biotechnology).

cDNA constructs and expression plasmids

Cloning of murine cytPTP ϵ cDNAs into the pcDNA3 vector (Invitrogen, Groningen, The Netherlands) has been described previously [19,24,25]. Expression plasmids encoding human RPTP ϵ , RPTP α , its catalytic inactive mutant forms Cys⁴⁴² → Ser (domain I) and Cys⁴⁴² → Ser/Cys⁷³² → Ser (domains I and II), and membrane-truncated cytPTP α have been described elsewhere [21,23]. Full-length human cytPTP ϵ was constructed from human RPTP ϵ by overlap extension PCR using the sense primer (5'-CGGGATCCGCCATGAGCAACAGGAGTAGCTTTTCCCGGCTACCTGGTTCAGGAAGCAGAGGAAAGCTG-3' including a *Bam*HI site, and the antisense primer (5'-ATGCA-GAACTTCCGGATGGTGTAGTCGACCAAAA-3') overlapping a unique *Sal*I site in RPTP ϵ . The coding sequence of the 12 amino acids unique to the cytoplasmic isoform is underlined (see Figure 1). For membrane attachment, the Lck tyrosine kinase dual acylation sequence [22] was attached to the N-terminal of human cytPTP ϵ by PCR amplification of the cDNA using a Lck sense primer (5'-CGGGATCCGCCATGGGCTGTGGCTGC-AGCTCACACCCGGAAGATGACTGGAGCAACAGGAGTAGCTTTTCC-3') and the antisense primer described above. Nucleotides that were fused to the wild-type cytPTP ϵ sequence are underlined (see Figure 1). The isolated PCR product (600 bp) was subcloned into the TA-vector (Invitrogen), digested with *Bam*HI and *Sal*I, and the subsequent *Bam*HI–*Sal*I fragment was used to replace the corresponding *Bam*HI–*Sal*I fragment in the cytPTP ϵ /pcDNA3 vector. The coding sequences of PTP ϵ fragments modified by PCR were verified by DNA sequencing.

Cell lines and tissue culture

Syrian BHK-IR cells were maintained at 36.5 °C under 5% CO₂ in Dulbecco's modified Eagles's medium (DMEM) containing 4.5 g/l glucose, 10% (v/v) fetal calf serum, 2 mM L-glutamine, 1 μ M methotrexate, 100 units/ml penicillin and 100 mg/ml streptomycin. Stable BHK-IR cell lines expressing PTPs were grown in the presence of 100 nM human insulin (Novo Nordisk), whereas the wild-type BHK cell line was grown in the absence of both insulin and methotrexate. Since the level of confluency of cell cultures influences the tyrosine phosphorylation level of cellular proteins [26] the cell lines were grown under standardized conditions [21].

Generation of PTP-expressing cell lines using insulin-dependent clonal selection in BHK-IR cells

Stable cell lines expressing both the insulin receptor and regulatory PTPs were established as previously described [21]. In brief, BHK-IR cells (50000/6 cm Petri dish) were incubated overnight and transfected with 6 μ g of cytomegalovirus-promoter-based expression plasmids containing cDNAs encoding the various PTP forms or the empty vector as a control. Eighteen hours after transfection, the cells were washed once and fresh medium was added. Either immediately or the following

Figure 1 Schematic representation of PTP ϵ and PTP α constructs

(A) RPTP ϵ , wild-type cytPTP ϵ and membrane-associated Lck-cytPTP ϵ . The transmembrane (TM) segment and the peptide sequence unique for the cytoplasmic PTP ϵ isoform (cyt) are underscored. The asterisk indicates the point where sequences of all forms of PTP ϵ become identical. Identical regions include the membrane proximal (D1) and distal (D2) PTP domains. The Lck tyrosine kinase dual acylation motif is shown in italics with sites for palmitoylation (Pal) and myristoylation (Myr) denoted. The extracellular domain and signal peptide are coloured grey. (B) RPTP α and cytPTP α control constructs. All PTP cDNA constructs were inserted into cytomegalovirus-promotor-based expression plasmids.

Table 1 Number of BHK-IR clones rescued by expression of differently localized PTPs

The score (rescue efficiency) was obtained by transfection of 50000 BHK-IR cells followed by an early (transfection 1) or late (transfection 2) onset of insulin selection. Following 10 days of selection, the numbers of insulin-resistant clones were counted. Note that all sets of transfections in the two experiments were conducted in parallel.

cDNA insert	Number of BHK-IR clones	
	Transfection 1	Transfection 2
Membrane-associated PTPs		
RPTP ϵ	159 (139, 156, 181)	268 (278, 257)
Lck-cytPTP ϵ	105 (105, 89, 120)	199 (162, 237)
RPTP α	169 (140, 173, 195)	233 (249, 217)
Intracellular PTPs		
CytPTP ϵ	22 (35, 10, 21)	38 (34, 31, 48)
CytPTP ϵ (mouse)*	7 (6, 2, 11)	8 (4, 9, 10)
CytPTP α	4 (3, 2, 6)	7 (6, 2, 11)
Controls		
RPTP α (inactive: Cys ⁴⁴² → Ser)	0 (0, 0, 0)	0 (0, 0, 0)
RPTP α (inactive: Cys ⁴⁴² → Ser/Cys ⁷³³ → Ser)	0 (0, 0, 0)	0 (0, 0, 0)
Empty vector†	0 (0, 0, 0)	0 (0, 0, 0)

* Mouse homologue; all other PTPs used in the study were derived from human cDNAs

† For comparison, all cDNAs were inserted into cytomegalovirus-promoter-based expression plasmids.

day, the cells were subjected to insulin stimulation (100 nM final concentration) and non-adherent cells were removed. This procedure was repeated for the following 10 days (each day non-adherent cells were removed and fresh medium containing insulin was added) allowing growth of stable-transfected insulin-resistant clones. Rescued clones were either subcloned individually to establish stable cell lines or stained with 0.5 % Crystal Violet and counted (Table 1). For the clonal-selection assay the indicated PTPs were transfected in parallel and each set of transfections was conducted in triplicate.

Subcellular fractionation

Cells were washed twice in ice-cold PBS and lysed in hypotonic buffer A [20 mM Tris/HCl (pH 7.4), 0.1 % 2-mercaptoethanol, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mg/ml aprotinin and 10 mg/ml leupeptin] by passing through a 27-gauge syringe needle. Complete cell lysis was confirmed by light microscopy. The lysate was centrifuged at 2000 *g* for 15 min at 4 °C in order to remove nuclei, and the supernatant was centrifuged at 100000 *g* for 30 min at 4 °C in a Beckman L8-80M ultracentrifuge. The supernatant was decanted (soluble cytosolic fraction) and the membrane pellet was solubilized in buffer A containing 1 % (v/v) Triton X-100. Following centrifugation at 20000 *g* for 30 min at 4 °C, the supernatant was decanted (membrane fraction). The cytosolic and membrane fractions were analysed by Western blotting for their PTP content. Detection of the insulin receptor served as a positive control for membrane localization.

Adhesion assay and characterization of cell morphology

Exponentially growing BHK-IR and BHK-IR/PTP cells were seeded at a density of 3×10^5 /well in 6-well plates and incubated in complete medium without insulin. After 24 h, the cells were washed once in PBS, and 3 ml of DMEM supplemented with 0.5 % fetal calf serum was added per well. Insulin (final concentration of 100 nM) was added to three wells/plate (+ insulin) whereas the other three wells served as controls (– insulin). After a further 24 h of incubation, phase-contrast micrographs of cells were recorded (with and without insulin treatment) and the number of adherent and non-adherent cells was determined

following three standardized washes (3×3 ml) with PBS. Non-adherent cell numbers were determined from the collected wash solutions and the adherent cells were trypsin-treated to obtain the cell number. Cell counts were determined in triplicate for each well using a Coulter counter.

Characterization of stable cell lines by Western-blot analysis

To compare the influence of PTP localization on the phosphorylation level of cellular proteins, exponentially growing cells were trypsin-treated and replated at a density of 200000 cells/well in 6-well plates containing 3 ml of medium without insulin. After 24 h the cells were washed once in DMEM containing 0.5 % fetal calf serum and were then serum-starved overnight in the same medium. Cells were treated with insulin at the indicated final concentrations for 30 min, and lysed in ice-cold SDS sample buffer [20 % (v/v) glycerol, 3 % (w/v) SDS and 3 % (v/v) 2-mercaptoethanol] premixed with buffer B [10 mM Tris/HCl (pH 7.4), 10 % glycerol, 100 mM NaCl, 10 mM pyrophosphate, 1 mM sodium orthovanadate, 5 mM iodoacetic acid, 2 mM PMSF, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin] containing 1 % Triton X-100. Samples were boiled for 5 min and resolved by SDS/PAGE (Novex NuPAGE™ precast 4–12 % Bis-Tris gels) by loading lysate corresponding to the same number of cells in the individual lanes. To assess the cell numbers, 6-well plates from each of the cell lines were grown in parallel with those used for immunoblotting. These plates were trypsin-treated and the cell number was determined using a Coulter counter. Immunoblots were visualized using HRP-linked goat anti-rabbit and goat anti-mouse antibodies (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and enhanced chemiluminescence (ECL®; Amersham Pharmacia Biotech, Uppsala, Sweden). Chemiluminescence was recorded using a digital camera chip with a linear dynamic range of 1:1000 (Fujifilm Imager LAS-1000) allowing quantification of protein expression levels using the Fuji-Image Gauge version 2.53 software. For immunoprecipitation, cells (grown in 10-cm dishes, at 90 % confluency) were lysed in buffer B [supplemented with 1 % (v/v) Nonidet P40] and centrifuged at 20000 *g* for 15 min at 4 °C. The supernatants (1 mg of total protein, 500 μ l final volume) were incubated for 3 h at 4 °C with the indicated agarose-conjugated antibodies (4 μ g of antibody/

mg of protein) and the beads were washed three times in lysis buffer A without vanadate, suspended in SDS sample buffer and analysed by Western blotting as described above.

RESULTS

The presence of identical catalytic domains in wild-type cytoplasmic and transmembrane PTP ϵ offers a unique opportunity to explore the role of subcellular localization as a determinant of specificity in signal transduction. We have previously developed an insulin-based selection system in BHK-IR cells in which the negative regulatory role of PTP ϵ and PTP α on insulin-mediated cell rounding and detachment can be measured [21]. To gain insight into the functional consequences of compartmentalization of PTPs we have characterized the efficiency by which naturally occurring cytPTP ϵ and RPTP ϵ attenuate insulin-induced effects in these cells. For comparison, and to further dissect the role of the transmembrane segment and extracellular RPTP ϵ domain, we targeted the intracellular cytPTP ϵ isoform to the plasma membrane using the Lck-dual acylation motif (Figure 1A). The selected PTPs [including differently localized PTP α control forms (Figure 1B)] were studied in two different ways: (1) using a clonal selection assay, and (2) in stable cell lines.

RPTP ϵ and cytPTP ϵ differ in their efficiency to generate insulin-resistant clones

To assess the influence of cytPTP ϵ and RPTP ϵ enzyme activity on activated insulin receptor signalling pathway(s), BHK-IR cells were transfected with expression plasmids encoding the indicated PTP ϵ and PTP α forms (Figure 1) followed by daily insulin stimulation and removal of non-adherent cells (see the Materials and methods section). The number of insulin-resistant clones (rescue efficiency) obtained after 10 days was taken as a measure of the efficiency of each PTP to generate stable-transfected clones. In contrast with traditional coexpression methodologies, the insulin-dependent selection system only allows for the detection of clones if the expressed protein inhibits signal transduction processes leading to cell detachment.

When testing our panel of PTPs in the clonal selection assay, the selection pressure exerted by insulin was applied either immediately after the transfection (Table 1, transfection 1) or 24 h after removal of the transfection medium (Table 1, transfection 2). In both experiments, RPTP ϵ opposed with high efficiency the anti-adhesive effects induced by insulin. In contrast, intracellular cytPTP ϵ was a poor suppressor of cell detachment. Importantly, since cytPTP ϵ is catalytically active in a manner similar to RPTP ϵ [27], the lower rescuing efficiency of cytPTP ϵ indicates a genuine difference between PTP ϵ isoforms in their ability to regulate cellular signalling. To analyse whether the transmembrane segment and/or the extracellular domain of RPTP ϵ were required for rescue, we targeted the cytPTP ϵ isoform to the plasma membrane using the Lck-dual acylation motif (Lck-cytPTP ϵ). Importantly, Lck-cytPTP ϵ restored the rescuing efficiency almost to that of wild-type RPTP ϵ (Table 1). Thus the efficiency in generating stable-transfected insulin-resistant clones appears to correlate with the presumed localization of PTP ϵ to the plasma membrane and is independent of the extracellular domain.

For further comparison, and consistent with the performance of the naturally occurring PTP ϵ isoforms, the closely related RPTP α rescued as efficiently as RPTP ϵ , whereas the membrane-truncated cytPTP α had minimal rescuing capacity. Catalytically inactive RPTP α and empty control vector did not rescue at all. Of note, the catalytically active forms of intracellular and membrane-located PTP ϵ and PTP α exhibited similar protein

Figure 2 Subcellular fractionation of stable BHK-IR/PTP cell lines

Hypotonic lysates from stable BHK-IR/PTP cell lines expressing the indicated PTPs were separated into soluble cytosolic (C) and membrane pellet (M) fractions by ultracentrifugation with total cell lysate (T) as control. The fractions were subjected to SDS/PAGE and analysed for PTP content (anti-PTP ϵ/α immunoblotting) using the insulin receptor (IR) as a positive control for membrane localization. Chemiluminescence was recorded using a digital camera chip as described in the Materials and methods section.

levels when transiently expressed (results not shown). Thus the observed rescue efficiencies are not due to differences in DNA quality or transfection efficiency, but reflect inherent differences in the abilities of the studied PTPs to modulate cell signalling pathways.

Subcellular localization of PTPs in stable cell lines

To study the subcellular localization of PTPs and to evaluate the efficiency of the membrane-targeting sequence of the Lck-cytPTP ϵ fusion protein we subsequently established a series of stable cell lines. These were characterized by both subcellular fractionation and immunofluorescence studies using the insulin receptor as a positive control for plasma membrane localization. Subcellular fractionation of total cell lysates demonstrated that wild-type cytPTP ϵ is predominantly a non-transmembrane molecule, consistent with previous studies [19,20] (Figure 2). Whereas both cytPTP ϵ and cytPTP α were detected for the most part in the soluble cytosolic fraction, Lck-cytPTP ϵ was found predominantly (approx. 70%) in the high-speed membrane pellet fraction, demonstrating that the targeting signal was functional. In contrast to the cytoplasmic enzymes, RPTP ϵ and RPTP α were detected exclusively in the membrane fraction along with the insulin receptor (Figure 2). The subcellular localizations of the various PTP ϵ and PTP α forms were confirmed by immunofluorescence; in particular, the Lck-dual acylation motif targeted the fusion protein (Lck-cytPTP ϵ) evenly to the plasma membrane (results not shown). Thus we conclude that the functional selection system in BHK-IR cells provides an experimental model to assess the influence of subcellular localization of PTPs on growth factor-induced signalling cascades.

Cells expressing PTP ϵ show a distinct growth pattern

Cell lines expressing either PTP ϵ or PTP α forms exhibit increased cell-to-cell contacts compared with parental BHK-IR cells. The clustering of BHK-IR/PTP ϵ cells is particularly obvious during growth at low cell density and is in sharp contrast with BHK-IR cells, which migrate and spread out during the same growth period (Figure 3A). Whereas the extracellular domain of RPTP μ has been shown to mediate homophilic binding and to increase cell-to-cell adhesion when expressed in insect cells [28], no function(s) are currently known for the extracellular domain of RPTP ϵ . However, since a similar growth pattern was detected in cell lines expressing either intracellular or membrane-associated PTP ϵ forms, the observed cell-to-cell clustering seems to be

Figure 3 Membrane localization of PTP ϵ is essential and sufficient to counteract completely insulin-induced cell rounding and cell detachment

Characterization of representative stable BHK-IR/PTP cell lines. (A) Growth pattern and morphological effects of insulin. (B) Cell-substratum adhesion efficiency. For the adhesion assay, the number of cells remaining bound to the substratum after 24 h of insulin stimulation and three standardized washes were determined and compared with the number of adherent non-stimulated cells. Results are presented as means $\pm$ S.D. ($n = 3$) for each set of experiments. Statistical analysis was performed using the Student's t -test and showed a significant effect of insulin on cell-substratum adhesion efficiency (* $P < 0.05$). wt, wild-type.

mediated by the enzymic activity itself and not by the short, highly glycosylated, extracellular domain of RPTP ϵ .

Cell-substratum adhesion efficiency depends on membrane localization of PTP ϵ enzyme activity

To explore the connection between subcellular localization of PTP ϵ and rescue efficiency (see Table 1), and to quantify the cell rounding effects of insulin (Figure 3A), we assessed the adhesion efficiency of the stable cell lines (Figure 3B). We have previously shown that detachment of the BHK-IR parental cell line starts as early as 1 h after addition of insulin [21], i.e. at a time point where significant changes are observed in the tyrosine phosphorylation level of a number of cellular proteins (see below). However, prolonged incubation periods are required to obtain reliable, quantitative data on more subtle differences in the detachment of different cell lines. We have, therefore, assessed the number of adherent cells remaining after standardized washing procedures for non-stimulated and insulin-stimulated cells incubated for 24 h (Figure 3B). Insulin stimulation decreased the number of adherent BHK-IR cells by 50 % compared with non-stimulated cells, whereas insulin had no effect on wild-type BHK control cells. When cells expressing intracellular cytPTP ϵ and cytPTP α were challenged in this assay, a smaller yet significant portion (approx. 20 %) of cells detached compared

with non-stimulated cells. In contrast, insulin stimulation had no effect on cell adhesion in cell lines expressing Lck-cytPTP ϵ , RPTP ϵ or RPTP α (Figure 3B), consistent with the absence of insulin-induced cell rounding (Figure 3A). Thus cell lines expressing cytPTP ϵ or cytPTP α are only partially resistant to the effects of insulin compared with cell lines expressing the corresponding membrane-localized forms.

Requirement for high cytPTP ϵ protein levels in maintaining insulin-resistant stable cell lines

The insulin-dependent selection system involves a functional balance between activated insulin receptor and opposing PTP activity. Therefore the minimal PTP level capable of maintaining insulin-resistant cell lines provides an added indication of the efficiency of the enzyme in counteracting cell detachment. In particular, a comparison of the expression levels between wild-type cytPTP ϵ and Lck-cytPTP ϵ in stable cell lines allows a direct quantitative assessment of the effect of membrane localization. The level of cytPTP ϵ protein was on average 25-fold higher than in cell lines expressing the membrane-attached Lck-cytPTP ϵ form (Figure 4). Thus membrane association was sufficient to reduce the minimal protein expression level that permitted growth of adherent cells in the presence of insulin. Likewise, substantially lower amounts of RPTP ϵ compared with cytPTP ϵ were sufficient

Figure 4 Requirement for high cytPTP ϵ protein levels in maintaining insulin-resistant cell lines

BHK-IR/PTP cell lines were established from the clonal-selection assay and their PTP ϵ protein level determined by anti-PTP ϵ immunoblotting. PTP protein levels were quantified using the Fujifilm imager LAS-100 as described in the Materials and methods section. The anti-(insulin receptor) (IR) blot verifies similar cell numbers in the lysates. Each lane contains protein from 2×10^5 cells. Representative BHK-IR/PTP cell lines are shown: BHK-IR/RPTP ϵ (1a, 2a, 3b, 4b and 7b); BHK-IR/Lck-cytPTP ϵ (2a, 3a, 7a and 6b); and BHK-IR/cytPTP ϵ (3a, 1a, 7a, 4a and 6b).

to generate stable cell lines (Figure 4). Similar results were obtained on analysis of PTP α -expressing clones (results not shown). The fact that only cell lines expressing exceptionally high cytPTP ϵ or cytPTP α protein levels were detected probably reflects the need for a minimal concentration of cytoplasmic enzyme to interact with appropriate substrates at the plasma membrane.

Both PTP protein level and subcellular localization determine the steady-state phosphorylation level of the insulin receptor β -subunit

The inability of cytPTP ϵ and cytPTP α to maintain stable insulin-resistant cell lines at protein levels as low as those of Lck-cytPTP ϵ or RPTPs warranted further investigation. To explore whether the subcellular localization of PTPs influenced enzyme activity towards the transmembrane insulin receptor, the level of cellular phosphoproteins in stable cell lines was determined. Serum-starved cell lines were stimulated with either 5 or 100 nM insulin for 30 min and compared with non-stimulated cells using standardized protocols. Immunoblot analyses showed different tyrosine phosphorylation levels of several proteins, including the insulin receptor β -subunit (Figure 5).

When comparing cell lines expressing the differently localized PTP ϵ forms, RPTP ϵ and Lck-cytPTP ϵ were more effective than cytPTP ϵ in maintaining a low basal tyrosine phosphorylation level of the insulin receptor β -subunit despite their markedly lower PTP protein levels (Figure 5, non-stimulated cells). However, upon insulin stimulation there was no substantial difference in the phosphorylation level of the insulin receptor β -subunit in the different cell lines, suggesting that the high cytPTP ϵ protein level was able to compensate for the lower accessibility of the cytoplasmic enzyme to the insulin receptor.

To assess the direct effect of membrane localization in determining PTP activity towards the insulin receptor β -subunit we next compared cell lines expressing similar PTP protein levels. Since relatively low levels of both Lck-cytPTP ϵ and RPTPs efficiently inhibit the signalling pathway(s), leading to cell detachment (see Table 1), the clonal insulin-dependent selection assay did not generate stable cell lines expressing these PTPs at the high levels required for rescue by cytoplasmic PTPs (see

Figure 5 Phosphorylation level of the insulin receptor depends both on PTP expression level and subcellular localization

Analysis of phosphotyrosine-containing proteins in stable BHK-IR cell lines overexpressing PTP ϵ and PTP α forms. (A) Anti-phosphotyrosine (pY) immunoblot of serum-starved cells untreated or treated for 30 min with 5 or 100 nM insulin. The molecular-mass marker proteins, insulin receptor β -subunit (IR β) and phosphoproteins pp35, pp70 and pp125 are indicated. (B) The expression level of insulin receptor verifies similar cell numbers in the lysates (approx. 8×10^4 cells/well). (C) Immunoblot detection of PTP ϵ and PTP α protein levels. The lower amounts of RPTP ϵ and Lck-cytPTP ϵ compared with cytPTP ϵ were typical for cell lines generated by the functional insulin-dependent selection system (see Figure 4). In contrast, the high RPTP α protein level (comparable to that required for rescue by cytPTP α and cytPTP ϵ) was fortuitously identified in one stable BHK-IR/RPTP α cell line, allowing a direct comparison of the effect of membrane localization of these PTPs on the phosphorylation level of cellular proteins.

Figure 4). Fortuitously, one RPTP α cell line was identified with comparably high protein levels to that of the cytPTP α -expressing cell lines, allowing us to directly address the role of subcellular localization of these PTPs (Figure 5). Comparison of these cell lines revealed that membrane localization associated strongly with a lower steady-state phosphorylation level of the insulin receptor β -subunit. In non-stimulated cells and cells stimulated with low insulin concentrations (5 nM), the phosphorylation level of the insulin receptor β -subunit was 3–4-fold lower in the presence of RPTP α compared with cytPTP α . However, high insulin concentrations (100 nM) resulted in maximal phosphorylation of the insulin receptor β -subunit in a manner independent of the presence or subcellular localization of PTP α (Figure 5). Thus we conclude that the net phosphorylation level of membrane-associated substrates is determined by both the PTP protein level and the subcellular localization of PTPs.

PTP ϵ lowers the phosphotyrosine content of a limited set of cellular substrates

Tyrosine phosphorylation levels of several endogenous substrates were considerably reduced in rescued BHK-IR/PTP ϵ cell lines compared with parental BHK-IR cells. Thus insulin-stimulated tyrosine phosphorylation of two protein bands (75 and 80 kDa) was completely absent in BHK-IR/PTP ϵ cell lines indicating preferential recognition of protein substrates by PTP ϵ and/or modulation of a subset of specific signalling pathways (Figure 5). As previously described [21], a 35 kDa protein (pp35) exhibited markedly reduced levels of tyrosine phosphorylation, although a distinct insulin-dependent increase in its phosphorylation remained visible.

The 70 kDa tyrosine-phosphorylated protein band (pp70), which appears only in cytPTP ϵ , cytPTP α and Lck-cytPTP ϵ expressing cells (Figure 5), probably represents the tyrosine phosphorylated forms of these enzymes. Consistent with this, both Lck-cytPTP ϵ and cytPTP ϵ were found to be present in anti-phosphotyrosine immunoprecipitates (results not shown). Intriguingly, almost identical amounts of tyrosine phosphorylated cytPTP ϵ and Lck-cytPTP ϵ were observed in the corresponding cell lines despite the compensatory higher cytPTP ϵ protein level.

Finally, the action of endogenous PTP activity was evident from the immunoblots. Thus insulin promoted the dephosphorylation of a 120–130 kDa protein (pp125) in parental BHK-IR cells. In sharp contrast, this band was hyper-phosphorylated in PTP ϵ -expressing cell lines, suggesting that this protein may be a possible mediator of the PTP inhibitory response. Therefore we subsequently performed immunoprecipitation studies to identify the nature of this protein.

Cells expressing PTP ϵ are resistant to insulin-induced dephosphorylation of FAK

FAK pp125 has been shown to undergo tyrosine dephosphorylation upon insulin stimulation in Chinese hamster ovary cells overexpressing the insulin receptor [29]. Since we observed a similar rapid dephosphorylation of pp125 after insulin stimulation of parental BHK-IR cells, we examined the possibility that pp125 was in fact FAK. To this end, we immunoprecipitated FAK from untreated and insulin-treated cells and immunoblotted with anti-phosphotyrosine antibodies (Figure 6A). Consistent with our hypothesis, compared with insulin-stimulated BHK-IR cells, a constitutively tyrosine phosphorylated 125 kDa protein was detected in immunoprecipitates from PTP ϵ -expressing cells. Stripping and re-probing the nitrocellulose membrane with polyclonal anti-FAK antibody identified the immunoprecipitated phosphoprotein as FAK and confirmed that equal

Figure 6 Cells expressing PTP ϵ are resistant to insulin-induced dephosphorylation of FAK

BHK-IR/PTP ϵ cell lines were serum-starved overnight and subsequently incubated for 30 min in the absence or presence of 100 nM insulin. (A) Cell lysates were subjected to immunoprecipitation (IP) with anti-FAK polyclonal antibody, and the resulting immunoprecipitates were resolved by SDS/PAGE and immunoblotted with either HRP-conjugated anti-pTyr (4G10) monoclonal antibody (top panel) or anti-FAK monoclonal antibody (bottom panel). The positions of FAK and tyrosine-phosphorylated FAK (pp125KDa) are indicated. (B) Cell lysate was prepared as above and subjected to immunoprecipitation with anti-Src monoclonal antibody followed by immunoblot analysis with either HRP-conjugated anti-phosphotyrosine (Py; 4G10) monoclonal antibody (top two panels) or anti-Src and anti-FAK polyclonal antibody (bottom two panels). Src was immunoprecipitated from 0.5 mg (BHK-IR and RPTP ϵ lanes) or from 2 mg (Lck-cytPTP ϵ and cytPTP ϵ lanes) of total protein.

amounts of protein were present in each lane. Importantly, the kinetics of FAK dephosphorylation in the BHK-IR parental cells, with > 95% reduction in phosphotyrosine content after 30 min, is consistent with the idea that hyper-phosphorylation of FAK in cells expressing PTP ϵ is not simply a result of the increased adhesiveness of these cells, but might be among the molecular events which cause it.

FAK (pp125) co-immunoprecipitates with Src in PTP ϵ -expressing cells

FAK is a well known substrate of Src and the major auto-phosphorylation site in FAK (Tyr³⁹⁷) provides a strong binding site for the Src SH2 domain [30]. Since recent studies have shown that RPTP α activates Src through dephosphorylation of its regulatory site (Tyr⁵²⁷) [31,32], and the intracellular segment of PTP ϵ exhibits 72% amino acid identity (89% similarity) with RPTP α , we next compared the tyrosine phosphorylation level of Src in parental BHK-IR cells and PTP ϵ -expressing cell lines under basal (–) and insulin-stimulated (+) conditions. Indeed,

immunoprecipitation of endogenous Src followed by SDS/PAGE and anti-phosphotyrosine and anti-Src Western-blot analysis (Figure 6B) identified reduced tyrosine phosphorylation of Src in PTP ϵ -expressing cells compared with BHK-IR cells. Furthermore, tyrosine-phosphorylated FAK (pp125) co-immunoprecipitated with Src under both basal and insulin-stimulated conditions in PTP ϵ -expressing cell lines, indicating a constitutively activated Src-FAK protein complex. In contrast, FAK did not co-immunoprecipitate with Src in insulin-stimulated BHK-IR cells (30 min stimulation), indicating a disruption of this complex compared with non-stimulated BHK-IR cells.

Based on the above results, we hypothesize that stabilization of the phospho-FAK-Src complex is, at least in part, responsible for the observed resistance to insulin-mediated cell rounding and detachment in PTP ϵ -expressing BHK-IR cells. In addition, our study identifies Src (or Src family kinases) as candidate physiological substrates of PTP ϵ .

DISCUSSION

The aim of the present study was to explore the importance of subcellular localization of PTPs in conveying specificity to intracellular signalling. The ability of PTP ϵ and PTP α to antagonize PTK signalling was investigated using a previously developed insulin-dependent clonal-selection assay and in BHK-IR/PTP cell lines [21]. In particular, three PTPs have been proposed as negative regulators of insulin signalling: PTP α [21], PTP-LAR (GenBank® accession number Y00815) [33] and PTP1B [34–36]. Recently, several different studies have provided significant support for PTP1B as a key regulator of the insulin receptor, including (1) combined enzyme kinetic and X-ray crystallographic analyses [37], (2) studies with PTP1B knockout mice [38,39], and (3) treatment of *ob/ob* and *db/db* mice with PTP1B antisense oligonucleotides (reviewed in [40]). Although our present and previous studies [21] show that the membrane-bound forms of PTP ϵ and PTP α , in this experimental setting, efficiently dephosphorylate the insulin receptor tyrosine kinase, it remains to be demonstrated that this is also the case in the major insulin target tissues *in vivo*. However, it should be emphasized that the BHK-IR/PTP selection system is used in the present study as a general model for PTP-mediated negative regulation of peptide hormone receptor signalling.

Several lines of evidence demonstrate a critical role for plasma membrane localization of PTPs in the negative regulation of hormone-induced cell detachment. Compared with RPTPs, the corresponding wild-type cytPTP ϵ and membrane-truncated cytPTP α were less efficient in: (1) generating insulin-resistant clones (see Table 1), (2) opposing insulin-induced cell rounding (see Figure 3A), and (3) mediating cell-substratum adhesion of stable BHK-IR/PTP cell lines (see Figure 3B). Importantly, anchoring of cytPTP ϵ to the plasma membrane via the Lck-dual acylation motif [22] restored high rescuing efficiency in the clonal-selection assay, and cell lines expressing Lck-cytPTP ϵ exhibited adhesion efficiencies compatible with that of RPTP ϵ .

A wide range of cell lines [15,41–44] exhibit growth-factor-induced morphological changes similar to those mediated by insulin in BHK-IR cells. As an example, activation of the epidermal growth factor receptor in A-431 carcinoma cells promotes cell rounding and cell detachment [45]. Furthermore, similar to our observations, expression of RPTP α , but not its catalytically inactive form, was associated with increased cell-substratum adhesion and resistance to epidermal growth factor-induced cell rounding [45].

The functional selection system in BHK-IR cells only allows isolation of stable cell lines if the signal transduction processes

leading to cell detachment are inhibited. Theoretically, this inhibition can be exerted both at upstream (i.e. the insulin receptor itself) or at downstream levels. Our findings demonstrate that the membrane-associated forms of PTP ϵ and PTP α are markedly more efficient than their cytoplasmic counterparts in mediating an inhibitory response. However, the fact that stable cell lines can be established with cytPTP ϵ indicates that lack of significant membrane-association can be compensated for by high expression levels. We speculate that the high cytPTP ϵ protein levels result in a sufficiently high concentration of the enzyme at the cell membrane to allow interaction with appropriate substrates. In accordance with this hypothesis, significantly lower amounts of PTP ϵ were sufficient to generate insulin-resistant cell lines when this cytoplasmic enzyme was artificially anchored to the plasma membrane via the Lck-dual acylation motif.

The influence of cytoplasmic compared with membrane-localized enzyme activity on cell signalling pathways has also been studied by others. Thus Klippel et al. [46] demonstrated that targeting of the catalytic subunit of phosphoinositide 3-kinase to the plasma membrane was sufficient to trigger multiple downstream signalling pathways, whereas expression of the wild-type cytoplasmic enzyme was not. However, expression of constitutively active cytoplasmic phosphoinositide 3-kinase evoked the same downstream responses as the membrane-targeted enzyme. This suggests, in accordance with our findings with cytPTP ϵ , that high levels of cytoplasmic enzyme activity and/or expression is able to compensate for the inherently lower capability of cytosolic enzymes to interact with membrane-associated signalling molecules.

The coupling between expression levels of regulatory molecules and rescue efficiency, as demonstrated in the BHK-IR/PTP ϵ cell lines, seems to be a general feature of functional selection systems. O'Reilly and Neel [5] have developed a rescue method in *Xenopus* embryos that parallels our system, since the efficiency of SHP2 variants to interfere with growth-factor-receptor signalling was quantified by titration of the minimal PTP protein level required for mesoderm induction. Using this methodology to analyse the structural determinants of SHP2 function, these authors concluded that the signalling efficiency of non-transmembrane PTPs is conferred by both intrinsic catalytic domain specificity and correct subcellular localization [5].

We find clear association between PTP membrane localization and low phosphotyrosine content of the insulin receptor. Fang et al. [47] have documented a similar effect of membrane localization on Src phosphorylation in transient coexpression experiments. When membrane attachment of the catalytic domains of PTP α , PTP λ and LAR was abolished, dephosphorylation of co-transfected v-Src substrate was likewise abrogated [47]. Also, localization of full-length PTP1B to the ER membrane accounts for a restricted substrate specificity in transient transfection studies [4], since truncated PTP1B, lacking its C-terminal ER-targeting sequence, is relatively non-selective [48].

Several PTPs are tyrosine phosphorylated in cells [25,47,49–53] and possess consensus binding sites for SH2-domain-containing signalling molecules. Previously, we and others have demonstrated that tyrosine phosphorylation of cytPTP ϵ [25], RPTP ϵ [25] and RPTP α [23,46,49,54] mediates association with the adaptor molecule growth-factor-receptor-bound protein 2 (Grb2). Furthermore, den Hertog et al. [49] have shown that phosphorylation of the C-terminal tyrosine residue in RPTP α is a direct measure of Grb2 binding in HEK-293 cells. Significantly, despite the 25-fold lower levels of Lck-cytPTP ϵ , we detected almost the same amount of tyrosine phosphorylated forms of

cytPTP ϵ and Lck–cytPTP ϵ in stable cell lines. Thus we speculate that phosphotyrosine-dependent Grb2 binding to PTP ϵ may play a role in counteracting insulin-induced cell detachment. In accordance with this, it was recently shown that mutation of the Grb2 binding site in RPTP α abolishes co-localization with focal adhesion plaques in NIH3T3 fibroblasts [55].

The present study suggests two possible molecular mechanisms by which PTP ϵ counteracts insulin-induced cell rounding and detachment in BHK-IR cells. First, PTP ϵ may dephosphorylate the autophosphorylated insulin receptor, thereby inhibiting signalling pathway(s) that modulate cell adhesion at the upstream level. Alternatively, or in addition, PTP ϵ may dephosphorylate (thereby activating) endogenous Src, which could subsequently phosphorylate FAK p125, a critical mediator of cell-adhesion and cytoskeletal remodelling [56].

With regard to the latter scenario, several groups have shown that association of Src with FAK, as evident from our co-immunoprecipitation result, promotes extensive phosphorylation of FAK on several tyrosine residues (including residues 407, 576, 577, 861 and 925) [57,58]. In accordance with this, we observe profound tyrosine phosphorylation of FAK in cells rescued by PTP ϵ expression. Importantly, the observed FAK–Src protein complex may be a key mediator of the PTP inhibitory response, since several molecules involved in cell adhesion (including Grb2, Grb7, p130Cas, paxillin and tensin) and molecules involved in insulin signalling (insulin receptor substrate-1, phosphoinositide 3-kinase and phospholipase C γ) have been shown to co-immunoprecipitate with FAK [59–64]. In view of recent results from the PTP α knockout mice [31,32] and previous studies identifying Src as a physiological substrate for PTP α [65,66], we speculate that the C-terminal regulatory phosphorylation site on Src (or Src family of kinases) may be the substrate of the closely related PTP ϵ . Moreover, such a mechanism is consistent with the observed growth pattern of PTP ϵ -expressing cell lines (i.e. increased cell clustering and cell-to-cell contacts compared with parental BHK-IR cells) and with earlier studies showing that Rous sarcoma virus-transformed cells are not well spread and display altered adhesion characteristics [67–70].

A distinct biological role for the extracellular domain of RPTPs has only been demonstrated for a few enzymes [28,71,72]. Unlike many RPTPs, the highly glycosylated extracellular regions of RPTP ϵ and RPTP α lack known adhesion motifs. Recently, the extracellular domain of RPTP α , which consists of 131 amino acids, was shown to associate with contactin in the nervous system [72]. However, at 27 amino acids, RPTP ϵ has the shortest extracellular domain of all known PTPs, and no soluble or surface anchored ligands to it have been reported so far. Since cell lines expressing either RPTP ϵ or Lck–cytPTP ϵ exhibit similar growth patterns, adhesion efficiency and resistance to insulin-induced cell rounding, the transmembrane and extracellular domains of RPTP ϵ apparently serve no role, other than providing membrane anchoring, in this experimental system. However, our experiments do not rule out a physiological role of the carbohydrate moiety of RPTP ϵ in, for example, protection from proteases or formation of ligand recognition/binding sites.

In summary, we have used naturally occurring PTP ϵ isoforms to evaluate the role of subcellular targeting of PTPs in controlling cellular signalling, using as a model system the ability of these PTPs to counter insulin-induced cell rounding and detachment. The pronounced difference in the efficiency with which PTP ϵ isoforms interfere with phosphotyrosine-based signalling pathways in BHK-IR cells demonstrates that substantial signalling specificity can be conferred through the transcriptional/translational control of subcellular localization of identical PTP catalytic domains *in vivo*.

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