A negative regulatory function for the protein tyrosine phosphatase PTP2C revealed by reconstruction of platelet-derived growth factor receptor signalling in *Schizosaccharomyces pombe*

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Abstract We have exploited reconstitution in the fission yeast *Schizosaccharomyces pombe* to investigate how activation of phospholipase Cγ (PLCγ) by the platelet-derived growth factor-β receptor (PDGFβR) is regulated by the SH2 domain-containing protein tyrosine phosphatase PTP2C (also known as SHP-2). When co-expressed in *S. pombe*, PTP2C abolished PDGFβR autophosphorylation as well as its ability to phosphorylate and activate PLCγ. Inhibition of PDGFβR signalling by PTP2C appears specific insofar that PTP1C, a close homologue of PTP2C, does not suppress activation of either PDGFβR or PLCγ. Surprisingly, an inactive PTP2C mutant (C459S), which dephosphorylates neither PDGFβR nor PLCγ, remains fully effective as an inhibitor of [^{3}H]inositol phosphate generation indicating that negative regulation is at least in part independent of catalytic activity. This contrasts with PLCγ activation by c-Src which, although blocked by active PTP2C, is not inhibited by the mutant PTP2C C459S. These observations indicate that in addition to a reported positive role relaying trophic signals, PTP2C can also exert a negative effect on the PDGFβR and its signalling to PLCγ.

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Key words: *Schizosaccharomyces pombe*; Phospholipase Cγ; Platelet-derived growth factor β receptor; Protein tyrosine phosphatase 1C; Protein tyrosine phosphatase 2C

1. Introduction

To mediate changes in cellular growth and differentiation, many trophic factors activate cell surface receptors by promoting subunit dimerisation and autophosphorylation at intracellular tyrosine residues. These provide high-affinity binding sites for *src* homology 2 (SH2) and the phosphotyrosine binding (PTB) domains within a set of signalling proteins which include the regulatory subunit of phosphatidylinositol 3′-kinase (p85 of PI3K), phospholipase Cγ (PLCγ), the GTPase-activating protein (GAP) of p21^{ras}, Src family tyrosine kinases, as well as adapter proteins such as SHC and the p21^{ras}-activating complex GRB2/SOS. These effector targets are then tyrosine phosphorylated and/or enzymatically activated leading to changes in cellular phosphorylation state and production of a range of second messenger molecules which together elicit an appropriate biological response [1–3]. Despite considerable progress in our mechanistic understanding of these events, one major outstanding question is how signalling from the receptor is terminated. Since tyrosine phosphorylation plays a central role in signal propagation it is however likely that protein tyrosine phosphatases (PTPs) play a key role in the process of down-regulation.

Over 75 PTPs have so far been identified and these can be categorised structurally into transmembrane and intracellular molecular species [4,5]. One class of intracellular PTPs possesses tandem SH2 domains within their N-terminus and appears to play a key role regulating signal transduction by cell surface receptors [6]. PTP1C (also known as SHP-1, HCP, SH-PTP1, SHP) for instance, is expressed predominantly in hematopoietic cells and suppresses responsiveness to IL3, steel factor, erythropoietin as well as mediating inhibition of several immunological signalling systems [6–12]. This is consistent with genetic evidence from two mouse mutants, *motheaten* and *viable motheaten*, where mutations within PTP1C result in excessive erythropoiesis and severe immune disregulation [13,14]. PTP2C (also known as SHP-2, Syp, PTP1D and SH-PTP2) is a second member of the SH2 domain subfamily and is expressed widely in many tissues [15–20]. PTP2C interacts with activated PDGF, EGF, HER2-neu and *c-kit* receptors as well as with insulin receptor substrate-1 [19–24] although surprisingly, this PTP appears to play a positive role mediating mitogenic signalling [25–27]. This also appears consistent with genetic analysis of the closely related *drosophila* gene *corkscrew* which acts upstream of Raf to transduce essential signals from the receptor tyrosine kinase *torso* during embryonic development [28,29]. What remains less clear from these studies however, are the targets of PTP2C catalytic activity and whether dephosphorylation of tyrosine residues could play a negative regulatory role in receptor-linked signalling.

We have reported recently use of the fission yeast *Schizosaccharomyces pombe* to reconstitute PDGFβ receptor (PDGFβR)- and c-Src-mediated phosphorylation and enzymatic activation of PLCγ [30]. In studies reported here we extend these findings to assess the role of PTP2C in this pathway.

2. Materials and methods

2.1. Strains, media and plasmid constructs

The host strain for *S. pombe* transformation, and the procedure for protoplast transformation were as described [30]. The bacterial strain for routine work was JM101TR (*supE, thi$^-$, srl::tn10, recA* Δ*(lac-proAB), [F′, traD36, proAB, lacIZ*ΔM*15]*) and standard media were used throughout. As described previously [30,31], cDNAs encoding rat PLCγ2 cDNA, wild-type and K602A point mutated mouse PDGFβ receptor, as well as human c-Src were subcloned into the thiamine-repressible *S. pombe* expression vectors pREP3, pREP4 and pREP7, respectively. In the appropriate host strain Sp31 (*h$^+$ leu1-32 ura4-D18 ade6-704 his5-303*) these vectors allow independent selection in media lacking leucine (pREP3), uracil (pREP4) or histidine (pREP7). Human Csk and where indicated Src cDNAs were expressed from the

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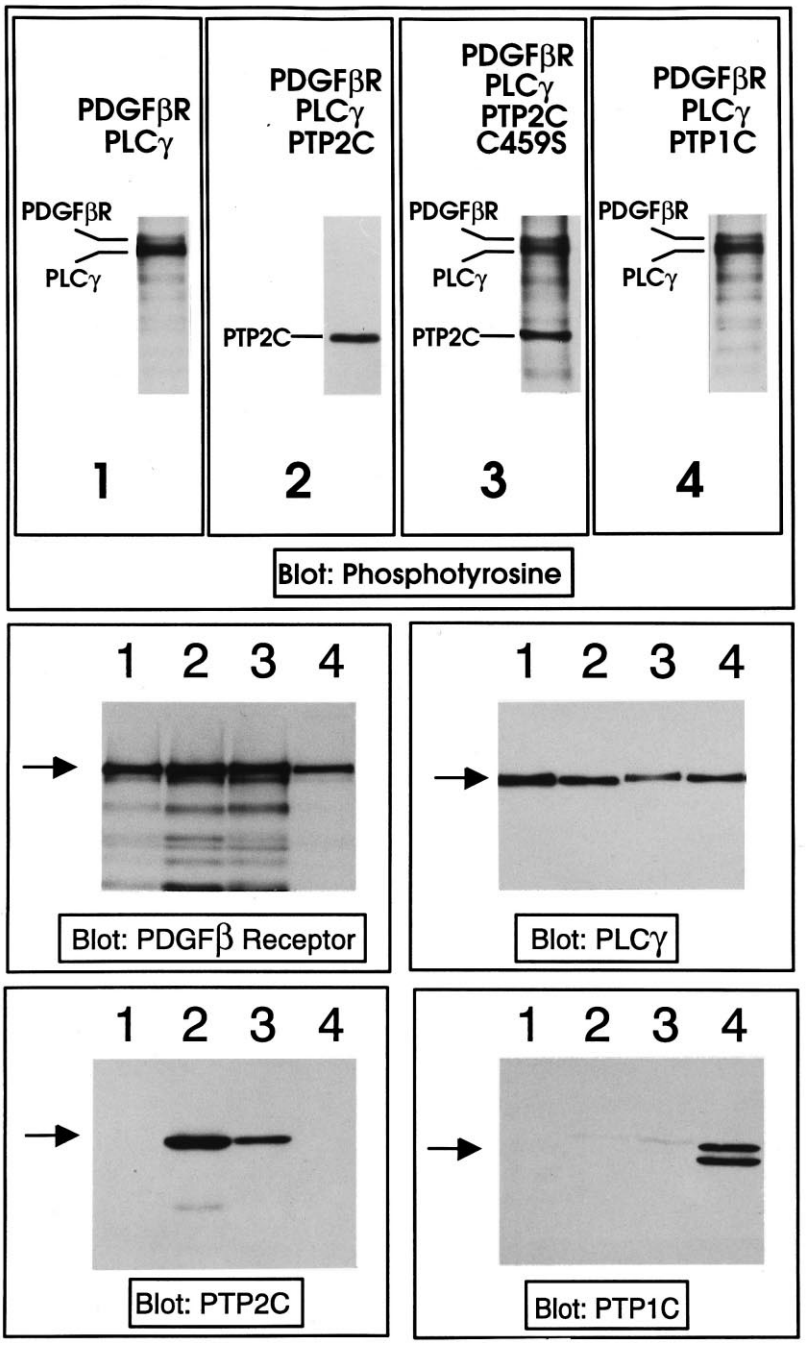

Fig. 1. PTP2C blocks tyrosine phosphorylation of the PDGFβR and PLCγ upon co-expression in *S. pombe*. Co-expression of PDGFβR, PLCγ2 and PTP2C or PTP1C in the fission yeast *S. pombe*. Western blot analysis was performed using crude homogenates prepared from yeast cells grown under inducing conditions for 18 h and expressing the mouse PDGFβR and rat PLCγ2 either alone (box/lane 1) or together with human PTP2C (box/lane 2), the inactive mutant PTP2C C459S (box/lane 3), or human PTP1C (box/lane 4). Samples were analysed using antibody specific for either phosphotyrosine, the PDGFβR, PLCγ2, PTP2C or PTP1C as indicated. The positions of immunoreactive proteins are indicated by arrows whereas bands corresponding to tyrosine phosphorylated PDGFβR, PLCγ and PTP2C are labelled where these are detectable. Phosphotyrosine boxes 1–4 represent the same exposure of the same Western blot with identical protein loading and are directly comparable. All antibodies failed to reveal immunoreactive bands in untransformed cells (see also [30]).

S. pombe adh promoter by subcloning into plasmids allowing selection in medium lacking leucine (pART1-leu), uracil (pART1-ura) or adenine (pART1-ade). PTP2C and PTP1C cDNAs were kindly provided by Dr. S.-H. Shen (Biotechnology Research Institute, Montreal, Quebec, Canada) and the coding sequences isolated as *Bam*H1-*Acc*I and *Cla*I-*Nru*I fragments, respectively, and subcloned into the *ade6*$^{+}$ vector pREP5. The catalytically inactive PTP2C point mutation C459S was constructed following using the mutagenic oligonucleotide CCAATTCCAGCACTAGAGTGCACCACGACC.

2.2. Yeast culture and sample handling

The growth, harvesting and homogenisation of yeast cells, as well as the labelling of yeast phosphoinositides using [^{3}H]inositol and subsequent isolation of [^{3}H]inositol phosphates, were all performed exactly as we reported previously [30]. Western analysis was also exactly as described [30] using antibodies from the following sources: c-Src (N16; Santa Cruz Biotechnology, Inc., Basel, Switzerland), Csk (Transduction laboratories, Nottingham, UK), PTP2C (C-18; Santa Cruz Biotechnology, Inc., Basel, Switzerland), PTP1C (#P17320;

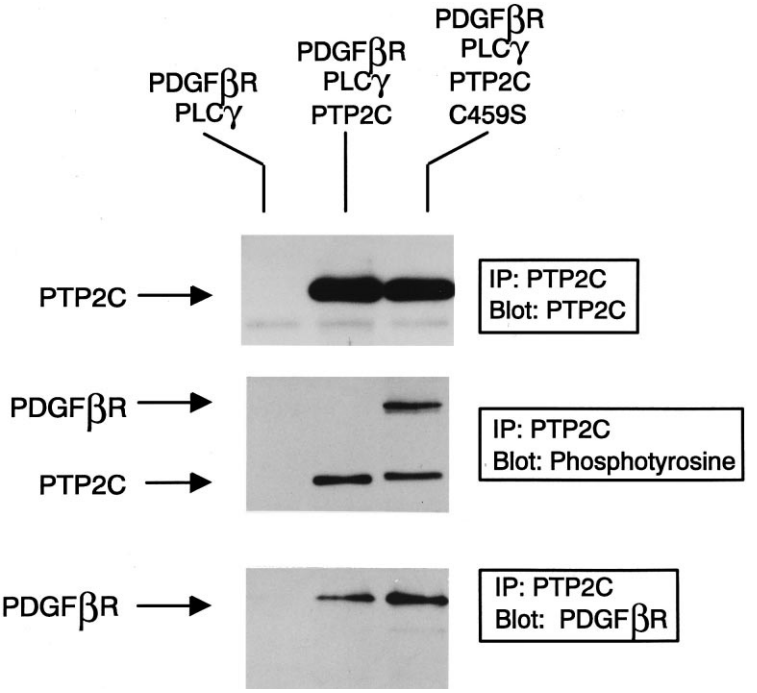

Fig. 2. PDGFβR co-immunoprecipitates with PTP2C from *S. pombe*. Lysates from yeast cells co-expressing the PDGFβR and PLCγ2 either alone or together with PTP2C or mutant inactive PTP2C C459S were subjected to immunoprecipitation using an antibody against PTP2C and protein A/G-Sepharose. Samples were analysed by Western blotting with antibodies specific for PTP2C, phosphotyrosine or PDGFβR as indicated. The positions of PDGFβR and PTP2C are indicated by arrows. Note that the PDGFβR is dephosphorylated only when coprecipitated with active PTP2C.

Transduction Laboratories, Nottingham, UK), PLCγ2 (C-19; Santa Cruz Biotechnology, Inc., Basel, Switzerland) and phosphotyrosine (4G10; Upstate Biotechnology, Inc., Lake Placid, USA). Antibodies specific for the mouse PDGFβ receptor and a second antibody recognising PLCγ2 were kindly supplied by Professor L.T. Williams (University of California, San Francisco, CA) and Professor Y. Homma (Fukushima Medical College, Japan), respectively. Antibody binding was detected using horseradish peroxide (HRP)-conjugated second antibody (Bio-Rad laboratories, Glattbrugg, Switzerland) or protein A/G-HRP (Pierce, Zurich, Switzerland) and ECL detection (Amersham, Zurich, Switzerland).

2.3. PTP2C and PLCγ2 immunoprecipitation

Crude homogenates were prepared and 200 μl aliquots mixed with 800 μl of ice-cold buffer L (1% NP40, 20 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 1 mM EDTA, 5 mM benzamidine, 10 μg/ml aprotonin, 10 μg/ml of leupeptin, 10 μg/ml of chymostatin and 1.0 mM PMSF at pH 7.5) followed by incubation on ice for 1 h. For immunoprecipitation samples were centrifuged at 100 000×*g* for 30 min and 800 μl of cleared lysate incubated with 20 μl of PTP2C or 10 μl of PLCγ2 specific antibody (as above) overnight at 4°C with mixing after which time 100 μl of a 1:1 (v/v) mix of protein A Sepharose and protein G Sepharose (50% w/v; Pharmacia Biotech., Uppsala, Sweden) was added and incubated for a further 2 h on ice with mixing. Beads were then sedimented by centrifugation and washed three times in 1.0 ml of buffer L followed by resuspension in 100 μl of SDS-PAGE sample buffer and heating at 95°C for 5 min.

3. Results

3.1. PTP2C dephosphorylates the PDGFβR and PLCγ

We have reported previously that mammalian PLCγ2 co-expressed with the PDGFβR in *S. pombe* becomes tyrosine phosphorylated and enzymatically activated to generate [^{3}H]inositol phosphates from prelabelled endogenous yeast membrane phosphoinositides [30]. It is of note that upon expression in *S. pombe* the PDGFβR appears to be constitutively active and insensitive to further stimulation with PDGF [30]. Using this system we have now investigated the regulatory actions of the human SH2 domain-containing protein tyrosine phosphatase PTP2C. When expressed in *S. pombe* PTP2C is immunodetectable as a protein of 68 kda and has no apparent effect on the levels of co-expressed PDGFβR or PLCγ2 (Fig. 1). The presence of PTP2C does however, abolish completely PDGFβR-dependent autophosphorylation as well as tyrosine phosphorylation of PLCγ2 (Fig. 1). Under these conditions the only protein undergoing extensive tyrosine phosphorylation in these cells is PTP2C itself. Immunoprecipitation experiments confirm the molecular identity of PTP2C as the major phosphoprotein under these conditions (Fig. 2) and is consistent with growth factor-dependent tyrosine phosphorylation of PTP2C reported previously in mammalian cells [19–22]. Dephosphorylation of the PDGFβR by PTP2C reflects tight interaction between these components as indicated by strong co-immunoprecipitation (Fig. 2). This contrasts with PLCγ2 which is not detectably precipitated using PTP2C antibody (not shown).

3.2. PTP2C blocks PDGFβR-dependent PLCγ activation

To assess the functional consequences of suppressing PDGFβR and PLCγ2 tyrosine phosphorylation, we measured generation of [^{3}H]inositol phosphates from prelabelled yeast cells [30]. Consistent with a critical role for tyrosine phosphorylation in this signalling pathway, PTP2C co-expression inhibits the generation of [^{3}H]inositol phosphates to near basal levels as observed when PLCγ2 is co-expressed with the inactive PDGFβR mutant, K602A (Fig. 3). To test the importance of PTP2C catalytic activity in the inhibition of

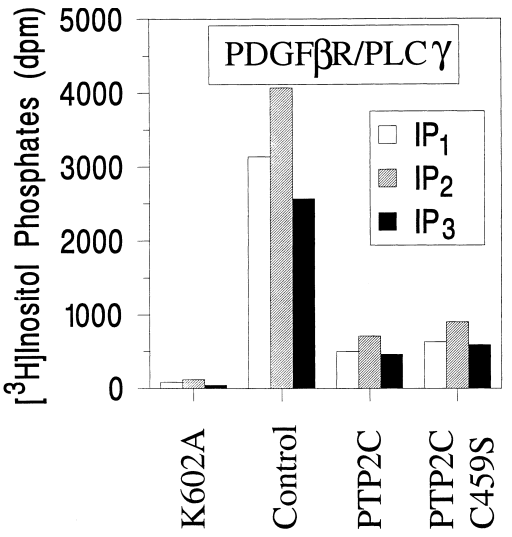

Fig. 3. PTP2C and inactive mutant PTP2C C459S block PLCγ2 enzymatic activation by PDGFβR. *S. pombe* expressing the PDGFβR and PLCγ2 either alone or together with PTP2C or the inactive mutant PTP2C C459S (as indicated) were grown under inducing conditions in the presence of [^{3}H]inositol for 18 h. Cells were extracted with $CHCl_3$-CH_3OH-HCl and [^{3}H]inositol phosphates isolated by anion-exchange chromatography. Radioactivity eluting as [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_3 is shown as disintegrations per minute (dpm) and is derived from a 6 ml yeast cell culture. PDGFβR catalytically inactivated by point mutation (K602A) fails to co-express PLCγ2 tyrosine phosphorylation in fission yeast [30] and demonstrates background production of [^{3}H]inositol phosphates. Bars represent the means of duplicate determinations and data are representatives of four separate experiments.

PDGFβR and PLCγ2 activation state we constructed the catalytically inactive point mutant C459S. As expected, this mutant is unable to dephosphorylate either PDGFβR or PLCγ2 (Fig. 1). Surprisingly however, PTP2C C459S remains fully effective as an inhibitor of PLCγ2 enzymatic activation (Fig. 3). As with wild-type PTP2C, the C459S mutant readily co-immunoprecipitates with the PDGFβ (Fig. 2). Together, these observations demonstrate that PTP2C is able to block PDGFβR-linked signalling to PLCγ2 but that this inhibition does not necessarily depend on tyrosine dephosphorylation.

3.3. PTP2C blocks PLCγ phosphorylation and activation by c-Src

To investigate further the negative regulatory role of PTP2C, we next analysed its influence on the ability of c-Src to stimulate PLCγ2 tyrosine phosphorylation and enzymatic activation [30]. For this study we constructed a yeast strain in which c-Src, Csk, PLCγ2 and PTP2C are all expressed in the same cell. The presence of each of these proteins was confirmed by Western blotting (Fig. 4). The distribution of phosphotyrosine-containing proteins (Fig. 4) shows that, in contrast to the results obtained with PDGFβR, c-Src or Csk enzymatic activity does not result in detectable tyrosine phosphorylation of PTP2C, nor does PTP2C alter the net level of c-Src tyrosine phosphorylation or indeed, c-Src enzymatic activity (not shown). Despite this, PTP2C is active in reversing the Src-induced tyrosine phosphorylation of PLCγ2, and as shown in Fig. 5, also abolishes hydrolysis of endogenous *S. pombe* [^{3}H]inositol-containing phospholipids. As expected, co-expression of the catalytically inactivated mutant PTP2C C459S is unable to prevent c-Src-dependent PLCγ2 tyrosine phosphorylation (Fig. 4). Interestingly, PTP2C C459S is tyrosine phosphorylated suggesting that Src and/or Csk can actually modify PTP2C but that when catalytically active this PTP is able to autodephosphorylate (Fig. 4). As anticipated, although in contrast to our observations with PDGFβR described above, catalytically inactive PTP2C C459S does not inhibit the ability of PLCγ2 to generate [^{3}H]inositol phosphates (Fig. 5) from prelabelled *S. pombe* membranes.

3.4. PTP1C is unable to dephosphorylate PDGFβR or its signalling to PLCγ

As a measure of specificity of PTP2C action, we assessed the regulatory actions of the PTP2C homologue PTP1C which is immunodetectable in yeast extracts as a doublet at 68 kda and 66 kDa (Figs. 1 and 4). In mammalian cells PTP1C runs as a protein of ~68 kda and we assume that the smaller of the two bands detected in yeast is a consequence of N-terminal degradation as the monoclonal antibody used for Western analysis recognises a peptide from the PTP1C C-terminal. In contrast to observations with PTP2C, when PTP1C was co-expressed together with PDGFβR and PLCγ2, there is no detectable decrease in tyrosine phosphorylation of either component (Fig. 1). Also unlike PTP2C, PTP1C is not detectably tyrosine phosphorylated by the PDGFβR (Fig. 1). Consistent with PTP1C inactivity against PDGFβR-dependent tyrosine phosphorylation, PTP1C was totally ineffective as an inhibitor of yeast phosphoinositide hydrolysis by activated PLCγ2 (data not shown). This does not reflect synthesis of inactive enzyme in *S. pombe* as PTP1C co-expression with c-Src substantially reduces both tyrosine phosphorylation (Fig. 4) and activation of PLCγ2 (not shown). Furthermore, as observed with PTP2C, PTP1C enzymatic activity is also evident by the complete reversal of Src-dependent tyrosine phosphorylation of multiple endogenous yeast proteins (Fig. 4).

4. Discussion

Fission yeast provides a convenient host in which to reconstitute interactions between mammalian signalling molecules [30,32–35]. Furthermore, since tyrosine phosphorylation appears not to be a feature of signal transmission in yeast and proteins containing the SH2 motif responsible for binding phosphotyrosine have not been identified, investigation of the mechanisms underlying tyrosine kinase-linked signalling is free from interfering endogenous yeast proteins. We have described previously activation of PLCγ in fission yeast in response to phosphorylation by both receptor (PDGFβR) and non-receptor (c-Src) tyrosine kinases [30] and in the present report we extend our findings to include the regulatory actions of the SH2-containing phosphatase PTP2C. Although most of the evidence to date indicates a positive signalling role for PTP2C (see Section 1), specific targets for PTP2C catalytic activity in vivo have not been identified and the question of whether tyrosine dephosphorylation by PTP2C plays a regulatory role in signalling remains unresolved. This is the question we have begun to address using the fission yeast. The principal conclusions of the present study are: (i) PTP2C acts selectively to dephosphorylate the PDGFβR but not c-Src; (ii) PTP2C dephosphorylates PLCγ modified by the PDGFβR and c-Src and blocks hydrolysis of endogenous inositol-containing phospholipids; (iii) the catalytically inactive

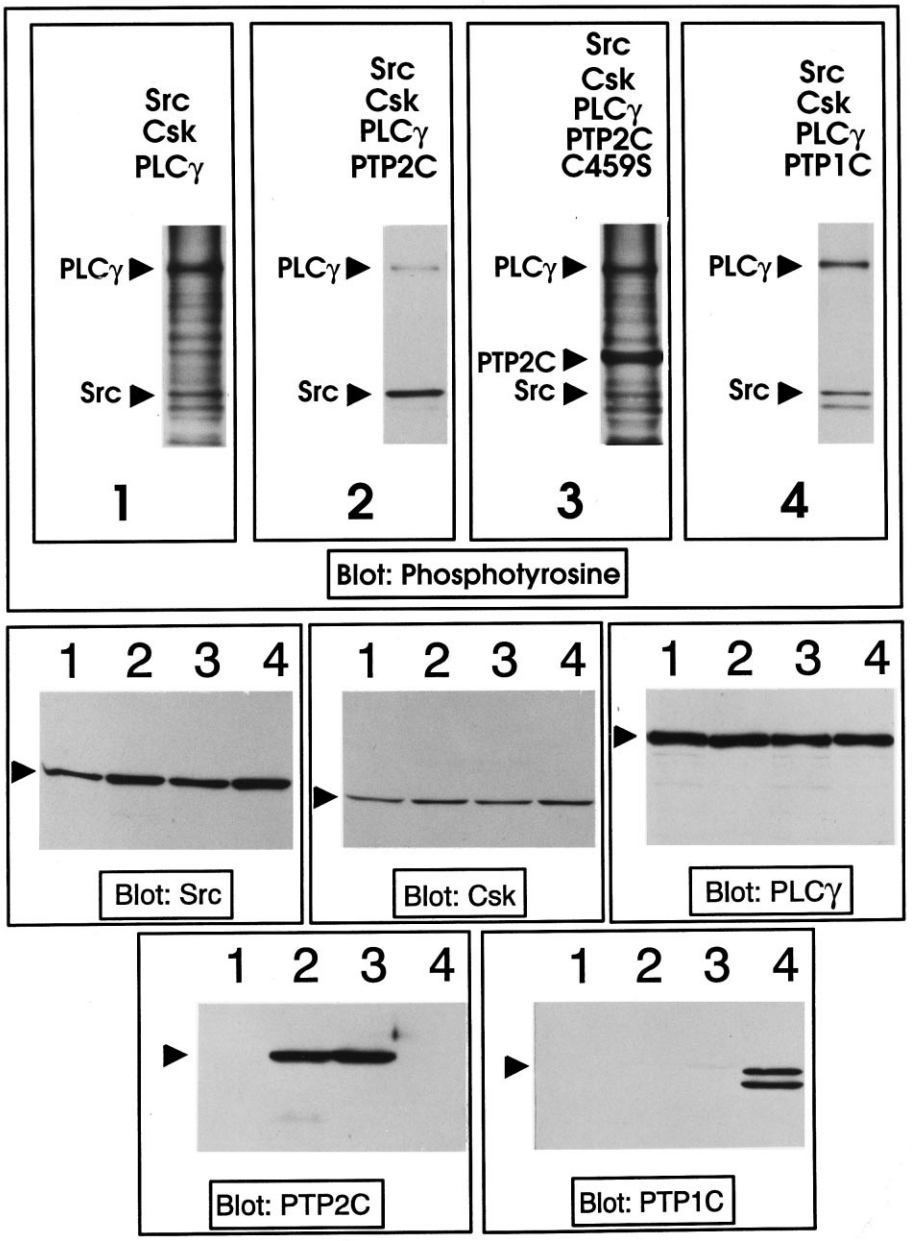

Fig. 4. PTP2C inhibits PLCγ tyrosine phosphorylation by c-Src. Co-expression of c-Src, Csk, PLCγ2 and PTP2C or PTP1C in *S. pombe*. Western blot analysis was performed using crude homogenates prepared from yeast cells grown under inducing conditions for 18 h and expressing human c-Src, human Csk and rat PLCγ2 either alone (box/lane 1) or together with human PTP2C (box/lane 2), inactive mutant PTP2C C459S (box/lane 3), or human PTP1C (box/lane 4). Extracts were probed using antibodies specific for either phosphotyrosine, Src, Csk, PLCγ, PTP2C or PTP1C. The positions of immunoreactive proteins are indicated by arrow heads whereas bands corresponding to tyrosine phosphorylated PLCγ, Src and mutant inactive PTP2C are labelled where these are detectable. Phosphotyrosine boxes 1–4 represent the same exposure of the same Western blot with identical protein loading and are directly comparable. All antibodies failed to reveal immunoreactive bands in untransformed cells (see also [30]).

mutant PTP2C C459S also inhibits PLCγ activation by PDGFβR but not by c-Src; and (iv) PTP1C does not regulate PDGFβR signalling revealing distinct substrate preferences for these two enzymes within the intracellular environment of fission yeast.

It is of note that although experiments in mammalian cells have indicated a positive signalling role for PTP2C [25–27], our observations in *S. pombe* are in agreement with recent reports showing that purified PTP2C dephosphorylates PDGFβR immunoprecipitated from activated mammalian cells [36] and that it displays substrate specificity for phosphopeptides corresponding to the C-terminal of the PDGFβR [37,38]. Negative control of the PDGFβR is also consistent with the slowed dephosphorylation of a point mutated PDGFβR (Y1009F) unable to interact with PTP2C following cellular stimulation [36]. Significantly, recent experiments using fibroblasts derived from mice homozygous for a targeted mutation in PTP2C also reveal negative regulatory control of PDGFβR tyrosine phosphorylation as well as its downstream signalling to MAP kinase activation [39]. Together, these observations indicate that in addition to mediating positive signals underlying trophic responses in some cells, PTP2C can

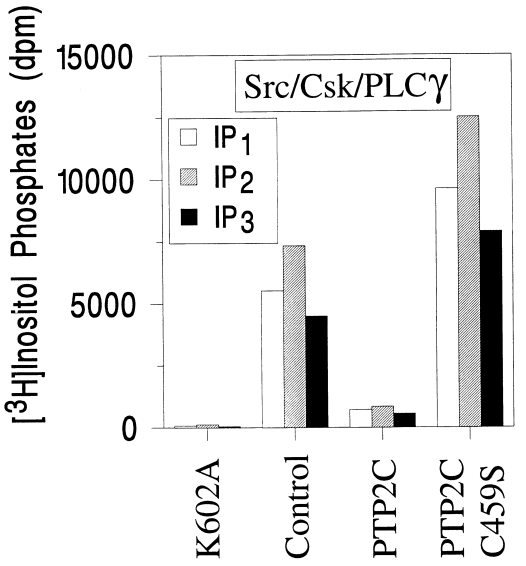

Fig. 5. PTP2C, but not inactive mutant PTP2C C459S, blocks PLCγ activation by Src. *S. pombe* expressing human c-Src, human Csk and rat PLCγ2 either alone (Control) or together with PTP2C or inactive mutant PTP2C C459S (as indicated) were grown in the presence of [^{3}H]inositol for 18 h. Cells were extracted with $CHCl_3$-CH_3OH-HCl and [^{3}H]inositol phosphates separated by anion-exchange chromatography. Radioactivity eluting as [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_3 is shown as disintegrations per minute (dpm) and is derived from a 6 ml yeast cell culture. PDGFβR catalytically inactivated by point mutation (K602A) and co-expressed with PLCγ2 is shown to demonstrate background [^{3}H]inositol phosphate generation. Bars represent the means of duplicate determinations and data are representatives of four separate experiments.

also mediate feedback inhibition of receptor tyrosine kinase activation and suppress coupling to a subset of effector targets.

One surprising result emerging from our studies was that a catalytically inactive PTP2C point mutant (C459S) was fully effective at inhibiting PDGFβR-dependent activation of otherwise highly phosphorylated PLCγ2. This contrasts with PTP2C C459S inactivity against PLCγ2 tyrosine phosphorylated and activated by c-Src suggesting an action at the level of the PDGFβR. Consistent with this, the PDGFβR but not PLCγ2 co-immunoprecipitated with PTP2C C459S. One potential mechanism accounting for blockade of inositol phosphate generation is a competitive inhibition by PTP2C C459S of productive interaction of PLCγ2 with the PDGFβR. This may be expected as SH2 domains within PTP2C and PLCγ appear to bind adjacent and potentially overlapping phosphotyrosine residues (Tyr1009, Tyr1021) within the C-terminal of the PDGFβR [21,40–43]. Consistent with this model, EGF receptor mutations lacking C-terminal residues crucial for PLCγ interaction fail to mediate enzymatic activation of PLCγ despite its extensive tyrosine phosphorylation [44,45]. Moreover, binding of GAP and the p85 subunit of PI3K to the PDGFβR were shown recently to silence signalling to PLCγ activation [46]. Coincident interactions between the PDGFβR and a subset of effector targets including GAP, PI3K and PTP2C may therefore block effective receptor interaction with PLCγ and thus inhibit its enzymatic activation.

In summary, although PTP2C and its *drosophila* homologue *corkscrew* appear to play a role mediating positive trophic signals, our observations in *S. pombe* indicate that PTP2C may also have a negative regulatory action inhibiting the activated state of the PDGFβR itself as well as suppressing coupling to a subset of downstream effector targets.

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