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Identification of Phospholipase C gamma1 as a Protein Tyrosine Phosphatase mu Substrate that Regulates Cell Migration

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Abstract

The receptor protein tyrosine phosphatase PTPmu has a cell-adhesion molecule-like extracellular segment and a catalytically active intracellular segment. This structure gives PTPmu the ability to transduce signals in response to cell-cell adhesion. Full-length PTPmu is down-regulated in glioma cells by proteolysis which is linked to increased migration of these cells in the brain. To gain insight into the substrates PTPmu may be dephosphorylating to suppress glioma cell migration, we used a substrate trapping method to identify PTPmu substrates in tumor cell lines. We identified both PKCdelta and PLCgamma1 as PTPmu substrates. As PLCgamma1 activation is linked to increased invasion of cancer cells, we set out to determine whether PTPmu may be upstream of PLCgamma1 in regulating glioma cell migration. We conducted brain slice assays using U87-MG human glioma cells in which PTPmu expression was reduced by shRNA to induce migration. Treatment of the same cells with PTPmu shRNA and a PLCgamma1 inhibitor prevented migration of the cells within the brain slice. These data suggest that PLCgamma1 is downstream of PTPmu and that dephosphorylation of PLCgamma1 is likely to be a major pathway through which PTPmu suppresses glioma cell migration.

Keywords

Receptor protein tyrosine phosphatase; Phospholipase C; Protein kinase C delta; RACK1; Cell migration; Glioblastoma; Tyrosine phosphorylation

INTRODUCTION

Glioblastoma multiforme, also referred to as grade IV astrocytoma, is the most common primary brain tumor in adults. Glioblastomas are particularly devastating due to their highly dispersive nature, which makes them resistant to the most advanced current therapies [Furnari et al., 2007]. Therefore, understanding the mechanism of glioma dispersal is key to designing effective therapeutics that limit the spread of disease throughout the brain.

Dispersal of glioma cells into the brain parenchyma involves a series of interactions with both the extracellular matrix (ECM) and neighboring cells. These interactions and the biochemical events that follow, result in changes to tumor cell surface receptors and remodeling of the ECM by proteolysis [Furnari et al., 2007; Nakada et al., 2007; Rao, 2003; Sala et al., 2008; Teodorczyk and Martin-Villalba, 2010]. Gliomas overexpress growth factor receptor tyrosine kinases (RTKs) and their ligands, creating a situation for autocrine

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and paracrine stimulation of cell survival and migration [Furnari et al., 2007; Kanu et al., 2009; Nakada et al., 2007]. The pathways initiated in response to RTK activation have been well described and the role these signaling pathways play in tumor progression is established. In addition to the role of amplified and mutated RTKs in human cancers, receptor protein tyrosine phosphatases (RPTPs) are also implicated in regulating tumor progression [Ostman et al., 2006]. However, in contrast to the signaling downstream of RTKs, the signaling pathways and substrates downstream of most receptor tyrosine phosphatases remain largely unknown.

RPTPs are transmembrane PTPs. The type IIb RPTP subfamily member, PTP μ , has cell adhesion molecule-like domains in its extracellular segment and two phosphatase domains in its intracellular segment. Only the membrane proximal (first) phosphatase domain has been demonstrated to have catalytic activity as there is a naturally occurring mutation in the second phosphatase domain which renders it catalytically inactive. PTP μ is capable of mediating cell-cell adhesion [Brady-Kalnay et al., 1993; Gebbink et al., 1993] and neurite outgrowth [Burden-Gulley and Brady-Kalnay, 1999]. In addition, PTP μ regulates cadherin-dependent adhesion [Hellberg et al., 2002] and neurite outgrowth [Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007; Oblander and Brady-Kalnay, 2010]. Recently, we found that full-length PTP μ is downregulated in primary human glioblastomas by proteolytic cleavage generating both an extracellular and an intracellular fragment [Burden-Gulley et al., 2010; Burgoyne et al., 2009a; Burgoyne et al., 2009b]. While over-expression of full-length PTP μ protein reduces glioma cell migration [Burgoyne et al., 2009b], the presence of a proteolytically cleaved intracellular fragment of PTP μ , capable of translocating to the nucleus, increases glioma cell migration [Burgoyne et al., 2009b]. These results imply that the presence of full-length PTP μ suppresses migration.

Studies from our laboratory demonstrated that catalytic activity of PTP μ is essential for regulating PTP μ -mediated processes [Burden-Gulley and Brady-Kalnay, 1999; Ensslen-Craig and Brady-Kalnay, 2005; Oblander et al., 2007]. To gain a better understanding of PTP μ function, a number of direct PTP μ binding proteins and potential substrates have been identified including, RACK1, p120, IQGAP1 and BCCIP [Mourton et al., 2001; Phillips-Mason et al., 2006; Phillips-Mason et al., 2008; Zondag et al., 2000]. RACK1, receptor for activated C kinase, is a WD-40 repeat protein that binds to protein kinase C (PKC), src family kinases, phospholipase C γ 1 (PLC γ 1) and cyclic AMP-specific phosphodiesterase PDE4 family members, among other proteins [McCahill et al., 2002]. RACK1 is thought to function as a scaffolding protein to bring signaling proteins in close proximity to one another. Both the association of RACK1 with PKC δ and PKC δ activity are required for the PTP μ -dependent regulation of axonal migration [Ensslen and Brady-Kalnay, 2004; Rosdahl et al., 2002]. BCCIP and IQGAP1 are required for PTP μ -dependent axonal migration [Phillips-Mason et al., 2006; Phillips-Mason et al., 2008] and BCCIP has been identified as a substrate for PTP μ *in vitro* [Phillips-Mason et al., 2008].

The mechanism by which PTP μ is able to suppress glioma cell migration and dispersal is not known. In this study we performed “substrate trapping” experiments aimed at identifying PTP μ substrates involved in the regulation of cell migration. This experimental approach has proven to be successful in identifying substrates for other protein tyrosine phosphatases (methods reviewed in [Blanchetot et al., 2005]). In this manuscript, we determined that both PKC δ and PLC γ 1 are substrates of PTP μ *in vitro*.

PLC γ is a key regulator of cell migration downstream of RTK signaling [Piccolo et al., 2002; Wells and Grandis, 2003]. Phosphorylation on tyrosine residue 783 of PLC γ 1 is critical to its activation [Poulin et al., 2005; Sekiya et al., 2004; Yu et al., 1998]. To ascertain whether PLC γ 1 is a downstream target of PTP μ involved in suppression of glioma

cell invasion, we conducted brain slice assays using U-87 MG glioma cells in which PTP μ protein had been knocked-down using shRNA. Reduced PTP μ expression in U-87 MG cells results in an increase in cell migration and dispersal [Burgoyne et al., 2009a]. In this manuscript, we demonstrate that addition of a PLC γ 1 inhibitor reverses this phenotype, and reduces glioma cell migration. These data imply that PLC γ 1 is functionally downstream of PTP μ . We hypothesize that PTP μ dephosphorylation of PLC γ 1 on residue Y783 prevents PLC γ activation thus blocking PLC γ 1-activated remodeling of the cytoskeleton and therefore, migration of glioma cells.

MATERIALS AND METHODS

CELL CULTURE

A549 non-small cell lung carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in F12 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), and 2mM L-glutamine (Invitrogen) at 37°C and 5% CO₂. U-87 MG and LN-229 human glioma cell lines were obtained from ATCC and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. Sf9 insect cells (CRL 1711; ATCC) were maintained at 27°C in Grace's insect media (Invitrogen) supplemented with 10% fetal bovine serum and 1 μ g/ml gentamicin.

REAGENTS

The SK18 monoclonal antibody directed against the intracellular domain of PTP μ has been described previously [Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994]. A polyclonal antibody to PKC δ and a monoclonal anti-phosphotyrosine antibody (PY99) were obtained from Santa Cruz (Santa Cruz, CA). The polyclonal, phospho-specific antibody to Y311 of PKC δ was obtained from BioSource (Camarillo, CA). Monoclonal antibodies to PLC γ 1 and RACK1 as well as polyclonal, phospho-specific antibodies to Y783 and Y771 of PLC γ 1 were obtained from BD Transduction Laboratories (San Diego, CA). A monoclonal antibody to vinculin was purchased from Sigma (St. Louis, MO). U-73122, a specific inhibitor of PLC γ 1 activity, was purchased from Calbiochem (La Jolla, CA). Purified, active His-tagged PLC γ 1 was purchased from Calbiochem. Purified, active His-PKC δ , active Src and active GST-epidermal growth factor receptor (EGFR) were purchased from Upstate Biosciences (Lake Placid, NY).

EXPRESSION OF GST FUSION PROTEINS IN ESCHERICHIA COLI

The intracellular segment of PTP μ contains two phosphatase domains. Only the membrane proximal (first) phosphatase domain has been demonstrated to have catalytic activity. For our studies we used intracellular constructs of PTP μ containing the juxtamembrane domain and the first phosphatase domain. iPTP μ WT- Δ D2 and iPTP μ DA- Δ D2 GST constructs have been described previously [Phillips-Mason et al., 2006]. iPTP μ DA- Δ D2 contains a D1063A mutation in the first phosphatase domain. Plasmids containing the intracellular PTP μ GST fusion proteins (iPTP μ WT- Δ D2 and iPTP μ DA- Δ D2) or GST alone were expressed in *E. coli* under the regulation of the *lac* promoter. GST and GST fusion proteins were isolated from *E. coli* using glutathione Sepharose 4B beads (Amersham Biosciences). Catalytically active iPTP μ GST fusion proteins used in substrate trapping experiments were isolated as previously described [Phillips-Mason et al., 2006]. Briefly, bacteria were resuspended in 10ml of buffer (0.1 M NaCl, 10 mM Tris-HCl at pH 8.0, and 1 mM EDTA) and incubated on ice for 15 minutes. To lyse bacterial cells, 1 ml of 0.5 M EDTA, 1.1 ml of 20% Triton X-100, 55 μ l of 1M dithiothreitol, 10 μ l of β -mercaptoethanol, 100 μ l of 100 mM phenylmethylsulfonylfluoride, and 30 μ l of protease inhibitor cocktail (Sigma) was added to 10 ml of resuspended cells. Cells were sonicated and spun at 15,000 rpm for 25 minutes.

GST fusion proteins were isolated from the cleared supernatant using glutathione Sepharose beads. GST and GST-iPTP μ WT used for *in vitro* binding assays with PLC γ 1 and RACK1 (described below) were isolated in PBST (PBS, 1% Triton X-100, 1 mM benzamidine, 5 μ g/ml aprotinin and leupeptin and 1 μ g/ml pepstatin). Protein expression and concentration of GST proteins was determined by Coomassie stain using BSA as a protein standard.

SUBSTRATE TRAPPING EXPERIMENTS

A549, U-87 MG and LN-229 cells were grown to 85–95% confluence and treated with or without pervanadate (100 μ M) for 20 minutes (Sodium orthovanadate is activated with hydrogen peroxide to make the cell-permeable, tyrosine phosphatase inhibitor, pervanadate). Cells were collected by scraping into lysis buffer containing 20 mM Hepes at pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM benzamidine, 5 μ g/ml aprotinin and leupeptin, 1 μ g/ml pepstatin and 5 mM iodoacetic acid (IAA) to inhibit any endogenous phosphatases. Cell lysates were vortexed and incubated on ice for 15 minutes. Dithiothreitol was added to a final concentration of 10 mM and cell lysates were incubated on ice for an additional 15 minutes and then centrifuged at 3000 rpm for 3 minutes. Protein concentration of the cell lysates was determined using the BCATM Protein Assay Kit (Pierce, Rockford, IL) and equal amounts of protein (800 μ g–1 mg) were added to equal amounts of GST alone or GST fusion proteins adsorbed on glutathione Sepharose. Samples were rocked for two hours at 4°C, washed four times with lysis buffer without IAA and incubated with 2 $\times$ SDS sample buffer. One-third of the sample was resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting as described previously [Phillips-Mason et al., 2006]. The substrate trapping pull down assays were repeated a minimum of two times from each of the three cell lines used. Therefore, each protein described has been identified as a PTP μ interacting protein a minimum of six times.

IN VITRO KINASE AND PHOSPHATASE ASSAYS

Purified PKC δ was phosphorylated *in vitro* using Src tyrosine kinase as described below. PKC δ (2 μ g) was incubated with 15U of active Src kinase for 1.5 hours at room temperature in Src kinase buffer (50 mM Hepes at pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂ and 1 mM ATP). After the kinase reaction was complete, the entire reaction volume (40 μ l) was diluted 1:20 with phosphatase buffer (25 mM Hepes at pH 7.4, 50 mM NaCl and 5 mM DTT). Then, 250 ng of tyrosine phosphorylated PKC δ in 100 μ l of phosphatase buffer was incubated with 7, 15, or 30 μ g of active, GST-iPTP μ WT or GST-iPTP μ DA, on glutathione Sepharose, for 15 minutes at 30°C. The phosphatase assay was stopped by adding 100 μ l 2 $\times$ SDS sample buffer and incubating the samples at 95°C for five minutes. Approximately 6 ng of PKC δ from each sample was resolved by SDS-PAGE and transferred to nitrocellulose for immunoblot. Purified PLC γ 1 was phosphorylated *in vitro* by purified, active GST-tagged epidermal growth factor receptor (EGFR) as follows. PLC γ 1 (2 μ g) was incubated with 400 ng (101 U/mg) EGFR for 10 minutes at 30°C in EGFR kinase buffer (25 mM Hepes at pH 7.4, 10 mM MgCl₂, 2.5 mM MnCl₂, 0.1 mM DTT, 0.2 % Triton X-100 and 25 μ M ATP.) After the EGFR kinase reaction was complete, the entire reaction volume (40 μ l) was diluted 1:20 with phosphatase buffer (25 mM Hepes at pH 7.4, 50 mM NaCl and 5 mM DTT). The EGFR was removed from the reaction by a brief incubation (15 minutes) with 20 μ l packed glutathione Sepharose. Then, 250 ng of tyrosine phosphorylated PLC γ 1 in 100 μ l of phosphatase buffer was used in a phosphatase assay as described above for PKC δ .

IN VITRO BINDING ASSAY

Equal amounts of GST or GST-iPTP μ WT fusion protein adsorbed on glutathione Sepharose were incubated with purified His-tagged proteins (PLC γ 1 and RACK1) individually or in combination. Incubations were performed in PBS, 1% Triton X-100, 1 mM benzamidine, 5 μ g/ml aprotinin and leupeptin and 1 μ g/ml pepstatin. Samples were rocked at 4°C for two

hours, washed 4 times with buffer and incubated at 95°C for five minutes in 2× SDS sample buffer. One fourth of the sample was resolved by SDS-PAGE and transferred to nitrocellulose for immunoblot analysis. His-RACK1 was purified as follows. Full length RACK1 cloned into the pAcHTL-C baculovirus expression vector (BD Pharmingen, San Diego, CA) has been described previously [Mourton et al., 2001]. Baculovirus was produced using the RACK1-pAcHTL-C plasmid and the BaculoGold™ system (BD Pharmingen) and used to infect Sf9 insect cells. Forty-eight hours post infection, the cells were lysed and His-RACK1 was purified using the PrepEase™ His-Tagged Protein Purification Kit (USB, Cleveland, OH), following the manufacturer's protocol with one modification, 1% Triton X-100 was added to the lysis and wash buffers. Purity of the His-RACK1 protein was determined by Coomassie stain and verified by immunoblotting with a monoclonal antibody to RACK1.

CO-IMMUNOPRECIPITATIONS

A549 cells were grown to 90% confluency. Cells were washed twice with PBS and treated with the crosslinking reagent DSP (Pierce) at a final concentration of 1 mM in PBS for 10 minutes at room temperature. Tris-HCl, pH 7.5 was added to a final concentration of 50 mM for 15 minutes at room temperature. Cells were then washed once with PBS and lysed in 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM benzamidine and protease inhibitor cocktail. Samples were sonicated and centrifuged at 10,000 rpm for 5 minutes. Supernatants were saved and protein concentrations determined using the Bradford method. Immunoprecipitations were performed with equal amounts of supernatant protein (400µg) using protein A Sepharose (Pharmacia) pre-loaded with a rabbit polyclonal antibody directed to the extracellular MAM domain of PTPµ (494), or non-immune rabbit serum (NIRS). Samples were rocked at 4°C for 3–4 hours. Beads were washed four times with lysis buffer and heated to 37°C for 15 minutes and 95°C for 5 minutes in 2× SDS sample buffer. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by immunoblotting.

LENTIVIRUS PRODUCTION AND INFECTION

The lentiviral shRNA plasmid V2LHS_171008 targeting a region in the extracellular domain of human PTPµ was purchased from Open Biosystems (Huntsville, AL). A control lentiviral plasmid was a gift from Drs E. Johnson and R. Keri (Case Western Reserve University, Cleveland, OH). As described previously [Burgoyne et al., 2009a; Dull et al., 1998], VSV-G-pseudotyped lentiviral particles were produced by a triple transfection with shRNA constructs, pCMVΔR8.91 and pMD.G packaging plasmids into 293T cells. Viral particles were concentrated by ultracentrifugation and used to infect U-87 MG cells in the presence of 6 µg/ml polybrene. Three days post transfection, cells were used for brain slice assays described below. Knockdown of PTPµ was verified by immunoblotting with antibodies to PTPµ. Infection efficiency was determined by visualization of a green fluorescent protein (GFP) reporter by fluorescence microscopy.

BRAIN SLICE ASSAY

All brain slice experiments were performed in accordance with an approved protocol from the Case Western Reserve University Institutional Animal Care and Use Committee. Organotypic brain slice cultures were prepared according to previously described protocols with some modifications [Burgoyne et al., 2009a; Gogolla et al., 2006; Jacobsen and Miller, 2003]. For the brain slice assay, U-87 MG cells were infected with lentiviral constructs encoding either control or PTPµ shRNA containing a GFP reporter. Three days post infection, U-87 MG cells were treated with either 18 µM U-73122 (Calbiochem) or DMSO (vehicle) for 30 minutes at 37°C. Infected cells were then trypsinized and resuspended in DMEM containing 2 mg/ml Matrigel (BD Biosciences) at a concentration of 10⁵ cells/µl.

The cell suspension (0.5 μ l) was injected into the cortex of rat brain slices using a 2 μ l micropipettor as described previously [Burgoyne et al., 2009a]. Post injection, slices were incubated at 37°C for 48 hours and then fixed in 4 % formaldehyde overnight at 4°C. Fixed slices were washed with PBS, mounted on slides and viewed under a fluorescence microscope to assess cell migration into the brain tissue by GFP fluorescence. Images were captured with a 10 $\times$ objective using a Leica DMI 6000B automated inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) attached to a Retiga EXi camera (QImaging, Surrey, BC, Canada). The extent of migration of injected tumor cells into brain slices was quantitated using MetaMorph software (Molecular Devices, Downingtown, PA) as previously described [Burgoyne et al., 2009a]. The data is shown as area of migration in μ m² and represents 3 separate experiments with a minimum of 22 replicates. Error bars indicate standard error. Statistical significance was determined using an unpaired student's t-test.

RESULTS

PKC δ is a substrate of PTP μ

Substantial evidence from our laboratory suggests PKC δ is a substrate for PTP μ . PKC δ activity is required for both axonal migration of retinal ganglion cells on a PTP μ -coated surface and for PTP μ -mediated axon guidance of retinal ganglion cells [Ensslen and Brady-Kalnay, 2004; Rosdahl et al., 2002]. In addition, PTP μ regulation of E-cadherin-mediated adhesion involves PKC δ [Hellberg et al., 2002]. Therefore, to validate the “substrate trapping” procedure used in this manuscript, we chose to evaluate whether PKC δ could be identified as a PTP μ substrate using this method. We performed pull-down assays with two intracellular PTP μ -GST fusion proteins on glutathione Sepharose: wild-type (iPTP μ WT- Δ D2) and a substrate trapping mutant, with the catalytically essential aspartate residue mutated to alanine (iPTP μ DA- Δ D2). When the conserved aspartate residue of a PTP, which acts as a general acid during catalysis, is mutated to an alanine (DA), a “substrate trap” is created [Flint et al., 1997; Tiganis and Bennett, 2007]. The DA mutation generally only affects catalysis of the enzymatic reaction, rendering the mutant protein catalytically inactive, but still able to interact effectively with its substrates because the affinity for its substrate remains the same. We first used these intracellular PTP μ -GST fusion proteins in pull-down assays with lysates prepared from confluent A549 carcinoma cells. A549 cells were chosen for our initial experiments because they express high levels of endogenous PTP μ and have proved valuable for identifying other PTP μ interacting proteins [Phillips-Mason et al., 2006]. For this assay, A549 cells were treated with or without the membrane permeable tyrosine phosphatase inhibitor pervanadate. Pervanadate was used to increase the intracellular pool of tyrosine phosphorylated proteins, increasing the probability of identifying proteins that interact in a phosphorylation-dependent manner. During cell lysis iodoacetic acid, an irreversible phosphatase inhibitor, was used to irreversibly inhibit all the endogenous, cellular phosphatases. This inhibitor was then quenched with DTT, allowing the recombinant, PTP μ -GST fusion proteins to remain active during the pull-downs. Therefore, any dephosphorylation that occurred *in vitro* was a consequence of the PTP μ -GST fusion protein. Proteins associated with the PTP μ -GST fusion proteins were resolved by SDS-PAGE and immunoblotted with antibodies to PKC δ and RACK1. As shown in Figure 1, PKC δ associated with iPTP μ WT- Δ D2 and iPTP μ DA- Δ D2 independent of pervanadate treatment. RACK1, a known PTP μ binding partner, also associated with both PTP μ -GST fusion proteins in all samples. Neither PKC δ nor RACK1 associated with the GST control. These data are in agreement with our published data that PKC δ and RACK1 interact with PTP μ [Mourton et al., 2001; Rosdahl et al., 2002]. To determine whether PKC δ is a PTP μ substrate, the samples were immunoblotted with a commercially available antibody specific to phospho-PKC δ Y311. Phospho-PKC δ Y311 was observed only in the

lysate treated with pervanadate. Furthermore, phospho-PKC δ Y311 was detected only in pull-downs performed with the substrate trapping mutant, iPTP μ DA- Δ D2 and not the wild type construct, iPTP μ WT- Δ D2. These data demonstrate that PKC δ is a PTP μ substrate since wild type PTP μ was able to dephosphorylate PKC δ , but the catalytically inactive construct was not. A mobility shift in PKC δ can be seen in those samples treated with pervanadate, suggesting the shift is due to tyrosine phosphorylation.

It is known that, in addition to interaction via the catalytic pocket, substrates interact with their cognate enzymes using protein-protein interaction domains outside of the catalytic region. An increase of total protein binding to a DA mutant over a WT mutant is not necessarily expected. However, one only sees “trapping” of the tyrosine phosphorylated protein in the DA mutant pull down. This implies the WT protein has dephosphorylated the protein on tyrosine residue(s) but that the DA mutant is not capable of dephosphorylating the tyrosine residue(s).

To verify PKC δ is a substrate of PTP μ we performed an *in vitro* phosphatase assay using purified PKC δ , phosphorylated by the non-receptor tyrosine kinase Src. Figure 3A shows that WT PTP μ was capable of dephosphorylating PKC δ *in vitro*, as measured by immunoblot with an anti-phospho-tyrosine antibody. The DA catalytic mutant of PTP μ was not able to dephosphorylate PKC δ confirming the requirement of PTP μ catalytic activity. The *in vitro* data, along with the substrate trapping results and our previously published functional data implying PKC δ is downstream of PTP μ , provide strong evidence that PKC δ is a substrate of PTP μ .

PLC γ 1 is a PTP μ substrate

Identification of PKC δ as a substrate for PTP μ confirmed that the substrate trapping method was a valid tool to identify PTP μ substrates. Because RACK1 is an established PTP μ interacting protein [Mourton et al., 2001; Rosdahl et al., 2002], and the RACK1 binding protein PKC δ was found to be a PTP μ substrate, we investigated whether other RACK1 binding partners were also PTP μ substrates. PLC γ 1 interacts with RACK1 [Disatnik et al., 1994] and is a good candidate to regulate cell migration downstream of PTP μ since PLC γ 1 activation is commonly associated with the migratory and invasive activity of tumor cells [Kassis et al., 1999; Mouneimne et al., 2004; Peak et al., 2008; Piccolo et al., 2002; Teodorczyk and Martin-Villalba, 2010; Thomas et al., 2003; Turner et al., 1997]. As described above, lysates from confluent A549 cells treated with or without pervanadate were incubated with iPTP μ WT- Δ D2 and iPTP μ DA- Δ D2. We tested whether PLC γ 1 was associated with the intracellular PTP μ -GST fusion proteins by immunoblotting with a PLC γ 1 monoclonal antibody (Fig. 2A). PLC γ 1 associated with iPTP μ WT- Δ D2 and iPTP μ DA- Δ D2, but not with the GST control (Fig. 2A). These data demonstrate PLC γ 1 can associate with PTP μ in a complex. To determine if PLC γ 1 is a substrate for PTP μ , we analyzed the pull-downs for the presence of phosphorylated PLC γ 1 using the two commercially available phospho-specific antibodies, which recognize phosphorylated residues Y783 and Y771. We found that phospho-PLC γ 1 Y783 was observed in the lysate treated with pervanadate, as expected. Phospho-PLC γ 1 Y783 was detected only in the pull-down with the substrate trapping mutant and not the wild type PTP μ construct, demonstrating PTP μ is capable of dephosphorylating PLC γ 1 on this residue (Fig. 2A). Because phosphorylation of PLC γ 1 on Y783 has been shown to be critical to its activity [Poulin et al., 2005; Sekiya et al., 2004; Yu et al., 1998], these data imply that PTP μ phosphatase activity is able to modulate PLC γ 1 activation. The Y771 phospho-specific antibody was able to detect a substantial pool of phosphorylated PLC γ 1 in the lysate of pervanadate treated cells. However, this antibody was only able to detect trace amounts of the phosphorylated protein in the pull-downs suggesting that the Y771 phosphorylated protein does not interact with PTP μ . The role of Y771 in the regulation of PLC γ 1 is not

clear, but it has been shown to be dispensable for PLC γ 1 activation [Serrano et al., 2005]. As with PKC δ , there is a slight shift in the mobility of PLC γ 1 in the samples treated with pervanadate although the shift is not as apparent with PLC γ 1 due to its larger size. The slight decrease in PLC γ 1 protein associated with iPTP μ DA- Δ D2 in the presence of pervanadate was not consistent.

We once again confirmed the substrate trapping data using an *in vitro* phosphatase assay. In this assay, PLC γ 1 was phosphorylated by the epidermal growth factor receptor (EGFR) and used as a substrate for PTP μ . Figure 3B shows that with increasing amounts of WT PTP μ there is a clear decrease in the tyrosine phosphorylation of PLC γ 1 as well as a dramatic shift downward in the mobility of the protein, which is consistent with its dephosphorylation. The PTP μ DA catalytic mutant was not able to dephosphorylate PLC γ 1.

PTP μ and PLC γ 1 interact *in vitro* and in cells

In order for PLC γ 1 to be a *bona fide* substrate for PTP μ , PLC γ 1 and PTP μ should interact *in vitro* and in cells [Tiganis and Bennett, 2007]. To determine whether PTP μ and PLC γ 1 interact directly, we performed *in vitro* binding studies using purified proteins. We were also interested in determining whether RACK1 was required for the interaction, so we included purified RACK1 in the experiment. Figure 3C shows that the intracellular PTP μ -GST fusion protein (iPTP μ WT- Δ D2) is able to bind purified PLC γ 1 both in the absence and presence of RACK1. There is still binding of both RACK1 and PLC γ 1 to PTP μ when both proteins are included in the assay. In these experiments it does not appear that PLC γ 1 requires RACK1 to bind to PTP μ . However, RACK1 addition results in a complex of RACK1, PLC γ 1 and PTP μ .

To test whether endogenous PLC γ 1 interacts with endogenous PTP μ in cells, we conducted co-immunoprecipitations using lysates of confluent A549 cells that had been treated with the DSP cross-linking reagent. PTP μ was immunoprecipitated using an antibody that recognizes the extracellular domain of PTP μ (494). Full-length PTP μ migrates as a 200kDa band, while a proteolytically processed fragment of PTP μ migrates at approximately 100kDa. PLC γ 1 interacts with PTP μ in co-immunoprecipitations from A549 cells (Fig. 2B), demonstrating that these two proteins interact in cells.

PKC δ and PLC γ 1 from glioma cell lysates interact with PTP μ

To verify that PKC δ and PLC γ 1 are potential substrates of PTP μ in glioma cells, we conducted pulldown experiments using cell lysates from two different human glioma cell lines, U-87 MG and LN-229. PKC δ bound to iPTP μ WT Δ D2 and iPTP μ DA- Δ D2 in both cell lines in the absence or presence of pervanadate (Fig. 4). Phospho-PKC δ was trapped by iPTP μ DA- Δ D2 in both cell lines as determined by the phospho-specific PKC δ Y311 antibody (Fig. 4). Similar results were observed for PLC γ 1. While PLC γ 1 associated with both forms of PTP μ -GST fusion proteins in the presence or absence of pervanadate, only iPTP μ DA- Δ D2 was able to trap pPLC γ 1 Y783 (Fig. 4). Since phosphorylation of Y783 is essential for PLC γ 1 activity, these results suggest that PTP μ may regulate PLC γ 1 activity in glioma cells. As seen in the pull-downs with A549 cells, iPTP μ DA- Δ D2 has little or no ability to bind the phospho-Y771 form of PLC γ 1.

PLC γ 1 is downstream of PTP μ in the regulation of glioma cell migration

Using an *ex vivo* brain slice assay that simulates glioma cell dispersal *in vivo*, we previously determined that down regulating PTP μ protein in U-87 MG cells increased tumor cell migration [Burgoyne et al., 2009a]. Three days post-infection with either control or PTP μ shRNA containing a GFP reporter, U-87 MG cells were injected *ex vivo* into the cortex of adult rat brain slices. Cell migration through the 3-D matrix environment was measured by

following migration using GFP fluorescence. The assay was quantitated by measuring the average area of fluorescent cells that migrated away from the injection site in a given slice after 48-hours. Control shRNA-infected U-87 MG cells were not dispersive and remained at the injection site (Fig. 5A). Knockdown of PTP μ by shRNA, however, induced a significant dispersal of cells away from the injection site into the adult brain tissue (Fig. 5A), suggesting that loss of PTP μ protein expression correlates with increased migration and dispersal of the glioma cells as previously demonstrated [Burgoyne et al., 2009a].

To demonstrate that PLC γ 1 is a substrate for PTP μ *in vivo*, and a potential target for migration regulation by PTP μ , we tested whether U-73122, a specific inhibitor of PLC γ 1, had any effect on the PTP μ knockdown-induced dispersal of U-87 MG cells. Pre-treatment of U-87 MG cells expressing an shRNA directed against PTP μ with U-73122 blocked the cell migration induced by PTP μ knockdown in the adult brain (Fig. 5A). Quantitation of the migration data shows that knockdown of PTP μ induced migration nearly 4-fold over control. Pre-incubation with the PLC γ 1 inhibitor reversed this phenotype, causing an approximate 2-fold reduction of the migration induced by knocking down PTP μ expression (Fig. 5C). These data suggest that PLC γ 1 is downstream of PTP μ and that dephosphorylation of PLC γ 1 is likely to be a key event in the pathway by which PTP μ suppresses glioma cell migration. It is important to note that the decrease in migration seen with the U-73122 pre-incubation was not due to altered cell viability. Excess cells from the brain slice injections were plated in tissue culture dishes and observed for 48 hours. There was no difference in cell viability between the control (DMSO treated) and U-73122 treated cells (data not shown). Figure 5B shows PTP μ protein expression was reduced by 64% following infection with lentivirus containing PTP μ shRNA.

DISCUSSION

PTP μ has been shown to regulate the migration of highly invasive glioma cells. In order to understand the molecular mechanism by which PTP μ influences glioma cell migration, we set out to identify substrates of PTP μ . In this manuscript, we identify PKC δ and PLC γ 1 as PTP μ substrates. Following the guidelines outlined by Tiganis and Bennett to define a PTP substrate [Tiganis and Bennett, 2007], we determine that PTP μ directly interacts with PLC γ 1 and that PTP μ is able to dephosphorylate PLC γ 1 *in vitro*. Substrate trapping experiments show a catalytically inactive mutant of PTP μ is able to trap PLC γ 1 from cell lysates as detected by a phospho-Y783 PLC γ 1 antibody. We have not yet been able to demonstrate changes in the tyrosine phosphorylation status of PLC γ 1 in intact cells when either wild type or catalytically inactive PTP μ is overexpressed in glioma cell lines followed by stimulation with various growth factors. However, we do provide functional data that PLC γ 1 is required for PTP μ to suppress glioma cell migration. Together, the data presented here suggest PTP μ modulates migration by dephosphorylating PLC γ 1 on Y783, rendering the enzyme inactive and unable to induce the cytoskeletal changes necessary for migration.

PTP μ expression is dramatically reduced in brain tissue excised from highly dispersive brain tumors compared to normal brain or low-grade astrocytomas [Burgoyne et al., 2009a]. The non-dispersive glioma cell line, U-87 MG, endogenously expresses PTP μ . In both an *in vitro* scratch wound assay and in a 3-dimensional *ex vivo* brain slice migration assay, we found that reduction of PTP μ expression in U-87 MG glioma cells increased cell migration [Burgoyne et al., 2009a]. These data suggest that PTP μ expression suppresses glioma cell dispersal and migration, perhaps by transducing signals in response to cell-cell adhesion. The mechanism whereby full-length PTP μ protein is reduced in glioma cells was determined to be via proteolytic cleavage of PTP μ into an extracellular and an intracellular fragment [Burden-Gulley et al., 2010; Burgoyne et al., 2009a; Burgoyne et al., 2009b]. The extracellular fragment of PTP μ retains its adhesive capabilities within the tumor

microenvironment of gliomas. Its presence at the tumor edge has been exploited as a potential diagnostic marker for glioblastomas [Burden-Gulley et al., 2010]. The intracellular fragment of PTP μ is capable of translocating to the nucleus of glioma cells. Reduction of the intracellular fragment of PTP μ using shRNA and the use of a peptide inhibitor of PTP μ catalytic activity decrease glioma cell migration, demonstrating that the fragment remains catalytically active following cleavage [Burgoyne et al., 2009a; Burgoyne et al., 2009b]. Generation of an intracellular PTP μ fragment would preclude PTP μ from dephosphorylating PLC γ 1, which is typically localized to the plasma membrane and the leading edge of migrating cells.

Tumor cell migration away from the central tumor mass occurs in response to growth factor gradients. Growth factors bind receptor tyrosine kinases that are often amplified or mutated in human gliomas, such as EGFR and PDGFR [Kanu et al., 2009; Teodorczyk and Martin-Villalba, 2010]. Upon ligand binding, RPTKs dimerize and undergo autophosphorylation to generate a series of phosphorylated tyrosine residues [Ullrich and Schlessinger, 1990]. Several of the resulting phospho-tyrosine residues serve as docking sites for adaptor proteins containing SH2 domains [Schlessinger, 2000]. Among the several proteins recruited to RPTKs upon ligand binding is PLC γ 1 [Wells and Grandis, 2003], one of a family of enzymes that hydrolyses phosphatidylinositol (4,5) biphosphate (PIP2) to its components, inositol-triphosphate (IP3) and diacylglycerol (DAG) [Choi et al., 2007]. In response to RPTK activation, PLC γ 1 is recruited to the cell membrane and phosphorylated by the receptor's intrinsic tyrosine kinase activity. In response to growth factors, PLC γ 1 can be phosphorylated on Y771, Y783, Y1253 [Sekiya et al., 2004] with the pattern and degree of phosphorylation depending on the growth factor. Of the tyrosine residues that can be phosphorylated, only tyrosine phosphorylation on residue 783 has been shown to be critical for PLC γ 1 activity [Poulin et al., 2005; Sekiya et al., 2004; Yu et al., 1998]. However, it was shown recently that in response to antigen receptor activation, PLC γ 1 is phosphorylated on Y775 [Serrano et al., 2005] and phosphorylation of this residue along with Y783 is required for PLC γ 1-mediated responses in Jurkat cells. A role for PLC γ 1 in the migration of carcinomas in response to growth factors has been established [Kassis et al., 1999; Mouneimne et al., 2004; Peak et al., 2008; Piccolo et al., 2002; Thomas et al., 2003; Turner et al., 1997] and there is evidence that PLC γ 1 plays a role in mediating migration and dispersal in glioma cells as well [Bruce and Parsa, 1999; Sala et al., 2008; Teodorczyk and Martin-Villalba, 2010]. Yet, the mechanism by which PLC γ 1 promotes migration is not completely understood. It is known that tumor cell invasion or dispersal requires rearrangement of the tumor cell cytoskeleton to form migratory structures such as lamellipodia and filopodia [Ridley et al., 2003; Yamaguchi and Condeelis, 2007]. Recent data suggests PLC γ 1 contributes to the migratory phenotype by directly [Li et al., 2009] or indirectly activating Rac1 [Jones and Katan, 2007; Sala et al., 2008], inducing migratory structures such as membrane ruffles and lamellipodia.

Malignant glioblastomas are resistant to current therapies due to their dispersive nature. PTP μ has been shown to modulate glioma cell dispersal in both *in vitro* and *ex vivo* models. We have demonstrated that full length PTP μ protein expression at the cell surface suppresses migration. In highly dispersive tumors and glioma cell lines, PTP μ is constitutively cleaved to generate an intracellular fragment. When PTP μ is released from the cell membrane as a result of proteolysis, it changes the availability of its substrates. We hypothesize that the mechanism by which full length PTP μ suppresses glioma cell migration is by dephosphorylating and inactivating PLC γ 1 at the cell surface, thereby suppressing PLC γ 1's ability to initiate remodeling of the actin cytoskeleton. In keeping with this hypothesis, we have shown that the migratory phenotype of U-87 MG cells induced by PTP μ knockdown is blocked by a specific Rac1 inhibitor [Burgoyne et al., 2009a]. Our current data suggest PLC γ 1 activates Rac1 to promote migration in glioma cells and PTP μ

acts to suppress migration by dephosphorylating PLC γ 1 on residue Y783. Therefore, proteolysis of PTP μ in gliomas would result in PLC γ 1 Y783 phosphorylation leading to unchecked cell migration and subsequent dispersal. Future studies will test the hypothesis that PTP μ cleavage is a critical switch that alters the ability of PTP μ to dephosphorylate PLC γ 1 thus leading to increased Rac1 activation and the promotion of cell migration.

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Figure 1.

PKC δ is a substrate for PTP μ . A549 cells were treated with (+) or without (-) 100 μ M pervanadate (PV) for 20 minutes. Cells were lysed and equal amounts of protein were incubated with iPTP μ WT- Δ D2-GST, iPTP μ DA- Δ D2-GST or GST immobilized on glutathione Sepharose. Associated proteins were resolved by SDS-PAGE (8% for PKC δ and 10% for RACK1) and detected by immunoblot. PKC δ and RACK1 were detected in all the PTP μ pull downs. Phospho-PKC δ Y311 was detected only in the iPTP μ DA- Δ D2-GST pull down, indicating PKC δ is a PTP μ substrate. The RACK1 immunoblot was stripped and probed with an antibody directed against GST to show the relative amounts of GST fusion proteins used.

Figure 2.

PLC γ 1 is a substrate for PTP μ and interacts with PTP μ in cells. A549 cells were treated with (+) or without (-) 100 μ M pervanadate (PV) for 20 minutes. Cells were lysed and equal amounts of protein were incubated with iPTP μ WT- Δ D2-GST, iPTP μ DA- Δ D2-GST or GST immobilized on glutathione Sepharose. Associated proteins were resolved by SDS-PAGE (6%) and detected by immunoblot. PLC γ 1 was detected in all the PTP μ pull downs but phospho-PLC γ 1 (Y783) was detected only in the iPTP μ DA- Δ D2-GST pull down, and dephosphorylated by iPTP μ WT- Δ D2 indicating that PLC γ 1 is a PTP μ substrate (A). Confluent A549 cells were treated with the DSP cross-linking agent. Immunoprecipitations were performed using a polyclonal antibody to an extracellular epitope of PTP μ or non-immune rabbit serum (NIRS). Proteins were resolved by SDS-PAGE (6%) and immunoblotted for PTP μ and PLC γ 1 (B).

In vitro binding and dephosphorylation of purified proteins **Figure 3.**

In vitro binding and dephosphorylation of purified proteins. His-tagged PKCδ was phosphorylated by Src *in vitro* and used as a substrate for PTPμ. Dephosphorylation of PKCδ by PTPμ was assessed by incubating equal amounts of phosphorylated PKCδ with increasing amounts of iPTPμ WT-ΔD2-GST or iPTPμ DA-ΔD2-GST immobilized on glutathione Sepharose. PKCδ was resolved on an 8% SDS-PAGE gel and its phosphorylation status determined by immunoblot with an anti-phosphotyrosine antibody. WT PTPμ was able to dephosphorylate PKCδ whereas the DA mutant of PTPμ was unable to dephosphorylate PKCδ. A duplicate gel was probed for total PKCδ and the phosphotyrosine immunoblot was stripped and re-probed for GST to show the relative amounts of the PTPμ GST fusion proteins (A). His-tagged PLCγ1 was phosphorylated by Epidermal Growth Factor Receptor *in vitro* and used as a substrate for PTPμ. The ability of PTPμ to dephosphorylate PLCγ1 was assessed as described above for PKCδ. PLCγ1 was resolved on a 6% SDS-PAGE gel. The dephosphorylation of PLCγ1 by WT PTPμ is indicated by the reduction in PLCγ1 tyrosine phosphorylation and the increase in mobility of the dephosphorylated protein. The phosphotyrosine immunoblot was stripped and re-probed to show total protein (PLCγ1) and a duplicate gel was probed for GST (B). PLCγ1 and PTPμ interact directly *in vitro*. iPTPμ WT-ΔD2-GST or GST alone were immobilized on glutathione Sepharose and incubated with purified His-PLCγ1 and His-RACK1 singly (R, RACK1; P, PLCγ1) or in combination (B, both RACK1 and PLCγ1). Bound proteins were resolved by SDS-PAGE (6% for PLCγ1 and 8% for RACK1) and detected by immunoblot. Both PLCγ1 and RACK1 were demonstrated to bind iPTPμ WT-ΔD2-GST independently. There was binding between PLCγ1 and PTPμ even in the presence of RACK1. The RACK1 immunoblot was stripped and re-probed for GST to show the relative amounts of GST fusion proteins used (C).

Figure 4.

PKC δ and PLC γ 1 are PTP μ substrates in glioma cell lines. The human glioma cells lines U-87 MG and LN-229 were used in substrate trapping experiments. U-87 MG and LN-229 cells were treated with (+) or without (–) 100 μ M pervanadate (PV) for 20 minutes. Cells were lysed and equal amounts of protein were incubated with iPTP μ WT- Δ D2-GST, iPTP μ DA- Δ D2-GST or GST immobilized on glutathione Sepharose. Associated proteins were resolved by SDS-PAGE (6% for PLC γ 1, 8% for PKC δ and 10% for RACK1) and immunoblotted for the indicated proteins. PLC γ 1, PKC δ and RACK1 were detected in all the PTP μ pull downs. Phospho-specific antibodies against PKC δ (Y311) and PLC γ 1 (Y783) confirm PKC δ and PLC γ 1 are PTP μ substrates in glioma cells.

Figure 5. PLC γ 1 inhibition abrogates the glioma cell migration induced by PTP μ knockdown. U-87 MG cells were infected with lentivirus encoding either a control shRNA or PTP μ shRNA with a GFP reporter and injected into the cortex of adult rat brain slices. Control shRNA-infected cells remain in a tight cluster at the injection site whereas the PTP μ shRNA-infected cells disperse into the brain parenchyma. When PTP μ shRNA-infected cells are pre-incubated with U-73122 prior to injection, the cells no longer migrate. Images were taken 48 hours post injection (A). PTP μ shRNA is efficient at reducing PTP μ protein expression in U-87 MG cells. Cells were infected with either control shRNA or PTP μ shRNA containing lentivirus. Three days post-infection, cell lysates were prepared and resolved on a 6% SDS-PAGE gel followed by immunoblot with a monoclonal antibody to PTP μ . Infection with PTP μ shRNA reduced PTP μ protein expression by 64% based on densitometry (B). The PTP μ immunoblot was stripped and re-probed with an antibody to vinculin as a loading control. Quantitation of brain slice migration. Data from three separate experiments (with a minimum of 22 replicates) were quantitated and plotted as area of migration in μm^2 according to the average threshold area of fluorescent cell that migrated away from the injection site (C). The asterisk represents a statistically significant difference ($p < 0.001$).