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Pleiotrophin stimulates tyrosine phosphorylation of β-adducin through inactivation of the transmembrane receptor protein tyrosine phosphatase β/ζ

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Abstract

Pleiotrophin (PTN the protein, *Ptn* the gene) signals through a unique mechanism; it inactivates the tyrosine phosphatase activity of its receptor, the transmembrane receptor protein tyrosine phosphatase (RPTP)β/ζ, and increases tyrosine phosphorylation of the substrates of RPTPβ/ζ through the continued activity of a yet to be described protein tyrosine kinase(s) in PTN-stimulated cells. We have now found that the cytoskeletal protein β-adducin interacts with the intracellular domain of RPTPβ/ζ in a yeast two-hybrid system, that β-adducin is a substrate of RPTPβ/ζ, that β-adducin is phosphorylated in tyrosine in cells not stimulated by PTN, and that tyrosine phosphorylation of β-adducin is sharply increased in PTN-stimulated cells, suggesting that β-adducin is a downstream target of and regulated by the PTN/RPTPβ/ζ signaling pathway. β-Catenin was the first downstream target of the PTN/RPTPβ/ζ signaling pathway to be identified; these data thus also suggest that PTN coordinately regulates steady state levels of tyrosine phosphorylation of the important cytoskeletal proteins β-adducin and β-catenin and, through PTN-stimulated tyrosine phosphorylation, β-adducin may contribute to the disruption of cytoskeletal structure, increased plasticity, and loss of homophilic cell–cell adhesion that are the consequences of PTN stimulation of cells and a characteristic feature of different malignant cells with mutations that activate constitutive expression of the endogenous *Ptn* gene.

Keywords: Pleiotrophin; β-Adducin; Receptor protein tyrosine phosphatase β/ζ

Pleiotrophin (PTN the protein, *Ptn* the gene) is a highly conserved 136 amino acid growth promoting, differentiation inducing cytokine [1–4]. Pleiotrophin expression is important in different developmental pathways [5,6], responses to injury [7], and in the progression of many human tumors [8–10] (Chang et al., Submitted; Perez-Pinera et al. Submitted).

Pleiotrophin is over 50% identical in amino acid sequence with midkine (MK the protein, *Mk* the gene), the only other member of *Ptn*/*Mk* developmental gene family [3,8,11]. Its expression is extensively regulated in embryonic development in a temporal and cell type-specific manner primarily in nervous and vascular systems [1,3,4,7,10,12–14]. However *Ptn* gene expression is constitutive and limited to few cell types in adults unless it is up-regulated in inflammatory macrophages, fibroblasts, and endothelial and other cells at sites of injury [7].

Pleiotrophin stimulates diverse functional responses in different contexts. It stimulates proliferation of fibroblasts, endothelial cells, and epithelial cells in culture [1,3,10,15,16], and neurite outgrowth and differentiation responses in neonatal neuronal cells [1,2] and both

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endogenous PTN and exogenous stimulate process outgrowth and oligodendrocyte lineage specific differentiation responses in glial progenitor cells in primary culture [7,17]. Male mice into which a dominant negative *Ptn* gene has been introduced have striking apoptosis in early sperm development and Sertoli cells [18] and recently, *Ptn* gene expression has been demonstrated to be essential for synthesis of the enzymes of the catecholamine biosynthesis pathway [5] and of the different proteins of the renin–angiotensin pathway [6,19].

The *Ptn* gene also is a proto-oncogene [9]. Mutations that activate constitutive *Ptn* gene expression "switch" the phenotype of pre-malignant cells to a phenotype of high malignancy, a phenotype often associated with striking tumor angiogenesis (Chang, et al. Submitted) [8,10]. Furthermore, mutations that activate the endogenous *Ptn* and establish constitutive *Ptn* gene expression are frequent in many human tumors of different histological origins [20–24]. When PTN signaling is interrupted by a dominant negative inhibitor of PTN [21] (Chang et al., Submitted) or with ribozyme constructions [25,26], the malignant phenotype of these highly malignant cells reverts to the pre-malignant phenotype. Collectively, the data strongly support the conclusion that activating mutations of the endogenous *Ptn* gene in the premalignant cell not only are frequent but alone are sufficient to initiate the highly malignant phenotype in many human cancers; mutations activating the *Ptn* gene may be among the most important in the progression of human malignancies.

The studies in the manuscript were designed to pursue the mechanisms by which PTN signals many different cellular responses noted above. In previous studies, we demonstrated that the transmembrane receptor protein tyrosine phosphatase (RPTP)β/ζ (Fig. 1) transmits the PTN signal; this pathway of PTN signaling is unique, since PTN is the first natural ligand to be discovered for any of the transmembrane tyrosine phosphatases [27]. RPTPβ/ζ is an important protein that is believed to have diverse function; it has numerous chondroitin sulfate side chains, and is expressed predominantly in brain [28–30]. The extracellular domain of RPTPβ/ζ differs significantly from other RPTPs. It has a carbonic anhydrase domain at the extreme N-terminal region, a fibronectin type III domain, and "cysteine free" domain immediately before the transmembrane spanning domain. RPTPβ/ζ binds different extracellular matrix molecules [31,32] and binds to and presumably is the receptor recognizing the *Helicobacter pylori* toxin Vac A [33], but, whether Vac A initiates a downstream signal is not known. There are three alternative splice variants of RPTPβ/ζ [34], including a full-length transmembrane form, a short transmembrane form lacking the extracellular, membrane proximal "cysteine free" domain, and a soluble form containing essentially the entire extracellular domain known as 6B4 proteoglycan, or "phosphacan" [35]. The intracellular domain of RPTPβ/ζ is highly conserved with the intracellular domain of other transmembrane receptor protein tyrosine phosphatases. The proximal intracellular domain of RPTPβ/ζ (defined here as the D1 domain, residues 1663–2034, Fig. 1) contains the active tyrosine phosphatase site of RPTPβ/ζ and the catalytic cysteine required for tyrosine phosphatase activity is residue 1932. The membrane distal (D2) domain contains a second, but inactive, tyrosine phosphatase domain and the extreme C-terminal amino acids define a short hydrophobic sequence predicted to bind to PDZ domain-containing proteins [36]. It was shown previously that PTN enforces dimerization of RPTPβ/ζ and inactivates its catalytic activity [27], presumably through a conformational change in the D1 (active site containing) domain of RPTPβ/ζ that prevents substrates phosphorylated in tyrosine from gaining access to their active site; this mechanism of the conformational change and block of access to the active site was demonstrated when the crystal structure of RPTPα was first solved [37]. PTN is the first naturally occurring ligand to any of the RPTPs and thus the PTN/MK signaling pathway is unique.

β-catenin was the first substrate of RPTPβ/ζ to be discovered. Tyrosine phosphorylation of β-catenin is markedly increased in PTN-stimulated cells [27] and the increase in tyrosine phosphorylation of β-catenin in PTN-stimulated cells reduces its affinity for the cytoplasmic tails of the cadherins (Perez-Pinera et al., submitted) disrupts the link of the cadherins and β-cate-

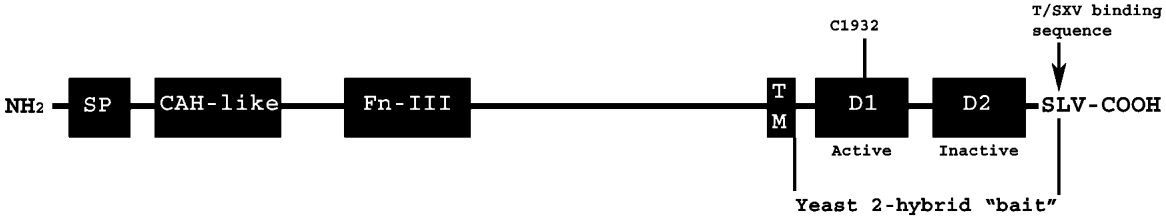

Fig. 1. Schematic representation of receptor protein tyrosine phosphatase (RPTP)β/ζ, SP, signal peptide; CAH-like, carbonic anhydrase-like domain; Fn-III, fibronectin type III domain containing condroitin sulfate; TM, transmembrane domain; D1, active tyrosine phosphatase domain (C1932-phosphatase catalytic residue); D2, inactive tyrosine phosphatase domain; SLV, C-terminal PDZ binding sequence. Yeast two-hybrid "bait", residues 1663–2314.

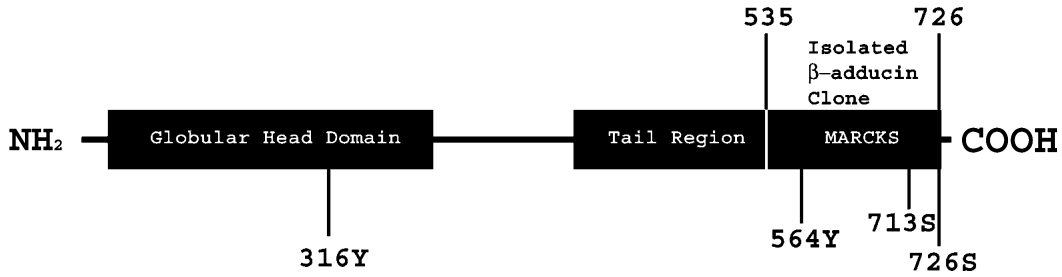

Fig. 2. Schematic representation of β-adducin. Shown is the globular head domain involved in the β-adducin multimer binding, the tail region containing the interactive clone of β-adducin identified in the yeast two-hybrid screen (residues 535–726), the myristoylated alanine-rich C-kinase substrate-like domain (MARCKS), and the consensus protein kinase C sites (residues 713S, and 726S), and the single putative tyrosine phosphorylation sites (residue 564Y).

nin through α-catenin with actin, disrupts adherent junctions, and reduces homophilic cell–cell adhesion (Perez-Pinera et al., submitted). These features are the early responses in PTN-stimulated cells [27] and are consistent with the known properties of β-catenin phosphorylated in tyrosines to disrupt these vital cytoskeletal complexes [38–41].

This study aimed to identify additional proteins interactive with RPTPβ/ζ and thus additional targets of the PTN/RPTPβ/ζ intracellular signaling cascade. The yeast two-hybrid system was used since it is a well-established and powerful tool to identify molecular interactions of proteins [42]. We screened a human fetal brain cDNA expression library with the intracellular domain of RPTPβ/ζ and identified the C-terminal 191 amino acids of β-adducin (see Fig. 2). β-adducin, like β-catenin, is an integral protein in cytoskeletal structure, and an important regulator of the integrity and fluidity of the cytoskeleton [43–47]. The association of the 191 amino acids fragment of β-adducin with the cytoplasmic domain of RPTPβ/ζ, therefore, suggested that β-adducin may be regulated coordinately by the PTN/RPTPβ/ζ signaling pathway and potentially β-adducin is a downstream target of PTN. The data generated now demonstrate directly that β-adducin is regulated through the PTN/RPTPβ/ζ signaling pathway.

Materials and methods

Yeast two-hybrid screen. AH109 competent yeast cells, the yeast expression vectors pGBKT7 and pACT1, and the yeast cells Y187 pretransformed human fetal brain MATCHMAKER cDNA Library were obtained from BD Biosciences Clontech (La Jolla, CA). The human fetal brain library was selected because of the previously observed high-level expression of RPTPβ/ζ and PTN in early brain development [12], suggesting that proteins in the PTN signaling pathway may be expressed in high levels in brain at that time. The full-length RPTPβ/ζ clone (Fig. 1) (GenBank Accession No. NM_002851) was a generous gift from H. Saito, Dana Farber Cancer Center, Boston, MA. The cytoplasmic domain of RPTPβ/ζ (residues 1663–2314) (see Fig. 1) was amplified using polymerase chain reaction (PCR) and subcloned into the yeast expression vector pGBKT7 for transformation of AH109 competent yeast cells. AH109 cells expressing RPTPβ/ζ residues 1663–2314 were confirmed by DNA sequencing analysis and Western blots of the induced AH109 cell lysates probed with anti-RPTPβ/ζ- specific antibodies (BD Transduction Laboratory, San Diego, CA). The confirmed strain was co-cultured with compatible Y187 yeast cells containing the pre-transformed human fetal brain library overnight in a shaker at 30 °C in 50 ml of YPAD yeast media. The mating yeast were plated on SD medium-stringency selection plates (SD/-His/-Leu/-Trp) and surviving colonies were re-plated on high stringency SD medium (SD/-His/-Leu/-Trp/-Ade). The colonies stained blue were tested for β-galactosidase activity with a colony filter-lift assay, the isolated library clones confirmed to be positive were purified, the cDNAs were sequenced, and the encoded proteins were identified by screening different standard databases.

To confirm the interaction of the isolated clones with the cytoplasmic domain of RPTPβ/ζ, AH109 cells were co-transformed with the vector pGBKT7-RPTPβ/ζ cytoplasmic domain and the vector pACT2 containing the brain library clone whose interaction was characterized as above. The isolated clones were confirmed for their ability to grow on the highest stringency selection plates and expression of α- and β-galactosidases.

Preparation of glutathione-S-transferase fusion proteins. The active site containing D1 domain of RPTPβ/ζ (residues 1663–2034) and an inactivated active site RPTPβ/ζ D1 domain mutant (residues 1663–2034, C1932S) were prepared by inserting the cDNA fragment encoding the human RPTPβ/ζ, amino acids 1663–2034 or 1663–2034 (C1932S) fused with glutathione-*S*-transferase (GST) to the bacterial expression plasmid pGEX-KG (Amersham Pharmacia, Piscataway, NJ). The GST fusion proteins have been termed GST-RPTPβ/ζ D1 and GST-RPTPβ/ζ D1 (C1932), respectively. The constructs (or GST alone) were expressed in BL-21 competent cells grown in 100 ml Luria–Bertani broth overnight, grown to middle log phase, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h, and lysed with 1% Triton X-100 in PBS with 0.1% phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin, and 1× Complete Protease Inhibition Cocktail (Roche Applied Sciences, Indianapolis, IN). The GST and GST fusion proteins were immobilized on 100 μl of glutathione–Sepharose-4B beads (Amersham Biosciences, Piscataway, NJ) and washed with 1% Triton X-100 in PBS. The GST-RPTPβ/ζ D1 fusion protein activity was tested by measuring its ability to dephosphorylate β-catenin phosphorylated in tyrosine to confirm it is an active tyrosine phosphatase. GST-RPTPβ/ζ D1 (C1932S) was shown to bind to, but not to dephosphorylate, β-catenin phosphorylated in tyrosine as previously described [27].

RPTPβ/ζ D1 and D1 (C1932S) GST "Capture" of β-adducin from cell lysates. To "capture" proteins interactive with RPTPβ/ζ D1 or RPTPβ/ζ D1 (C1932S), HeLa cell lysates were prepared and incubated at 4 °C with GST-RPTPβ/ζ D1 or GST-RPTPβ/ζ D1 (C1932S) coupled with glutathione–Sepharose-4B beads in PBS with Complete Protease Inhibition Cocktail tablets overnight, washed 5× with 0.25% Triton X-100 in PBS, boiled in SDS–PAGE sample buffer (25 mM

Tris–HCl, pH 6.8, 2.5% SDS, 2.5% glycerol, and 100mM DTT) for 5 min, resolved by SDS–PAGE, transferred to polyvinylidene difluoride membranes, probed with anti-β-adducin specific rabbit polyclonal antibodies (kindly provided by Dr.Vann Bennett, Duke University, Durham, NC), and incubated with anti-rabbit IgG HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and visualized using the chemiluminescence ECL Kit obtained from Amersham Biosciences, Piscataway, NJ.

Dephosphorylation of β-adducin phosphorylated in tyrosine by RPTPβ/ζ. Three 100-mm plates of HeLa cells were grown to 70% confluence in DMEM with 10% FBS, serum starved for 24 h, and stimulated with PTN 50 ng/ml (R&D Systems, Minneapolis, MN) for 30 min. The cells were lysed and samples were incubated with either GST-RPTPβ/ζ D1 or GST-RPTPβ/ζ D1 (C1932S) for 2 h in 25 mM Tris–HCl, pH 7.2, 10 mM DTT in PBS at 37 °C. After 2 h of incubation, β-adducin was immunoprecipitated from the lysates with 6 μg anti-β-adducin antibodies coupled to 50 μl of protein G–Sepharose beads and incubated overnight at 4 °C. Protein G coupled β-adducin was washed with 1% Triton X-100 in PBS, eluted, and analyzed in Western blots probed with anti-phosphotyrosine antibodies (BD Transduction Laboratory, La Jolla, CA). The blots were stripped and re-probed with anti-β-adducin to confirm the identity of β-adducin.

Results

Yeast two-hybrid screen: identification of the 191 C-terminal amino acid fragment of β-adducin

To seek proteins potentially targeted by PTN through its interaction and consequent inactivation of RPTPβ/ζ, a human fetal brain cDNA library was screened with the intracellular domain of RPTPβ/ζ as "bait" in a yeast two-hybrid screen as described under Materials and methods above. Eighty-eight yeast colonies were identified that grew under the stringent nutrient selective conditions and 12 of these grew under the most nutrient selective conditions. The inserts in each of the 12 colonies were sequenced and the DNA sequence of one of the isolated clones encoded a in-frame, 191 amino acid sequence that is identical to the 191 amino acid C-terminal sequence of human β-adducin (residues 535–726) (Fig. 2). Plasmids encoding the RPTPβ/ζ cytoplasmic "bait" domain and the 191 C-terminal fragment of β-adducin were used to transform A109 yeast cells. The cells formed large colonies under the most stringent nutrient selective conditions and expressed high levels of the reporter gene β-galactosidase, confirming that β-adducin associates with the intracellular domain of RPTPβ/ζ in the yeast two-hybrid system.

Analysis of the predicted sequence of the 191 amino acid C-terminal fragment identified a single putative tyrosine phosphorylation site LEE(Y564)KK. This putative phosphorylation site in β-adducin is nearly identical in amino acid sequence to a putative tyrosine phosphorylation site at the identical locus in the C-terminal region of both α-adducin and γ-adducin, and to a remarkably similar site in all vertebrate adducins screened, including *Gallus gallus* γ-adducin and *Danio rerio* α-adducin as shown in Fig. 3, suggesting that this putative tyrosine phosphorylation site is of high importance and perhaps needed for adducin functions, suggesting also it is this site in β-adducin that is recognized by the intracellular domain of RPTPβ/ζ.

β-adducin interacts with the active site containing (D1) domain of RPTPβ/ζ

The possibility that β-adducin is recognized by the active site D1 domain of RPTPβ/ζ was first tested. Residues 1663–2034 of RPTPβ/ζ, which contain the active site D1 domain of RPTPβ/ζ, and residues 1663–2034 (C1932S) of RPTPβ/ζ, which contain the inactivated catalytic cysteine (1932) active site mutant D1 domain of RPTPβ/ζ, were fused at their C-termini to glutathione-*S*-transferase (GST), coupled to glutathione–Sepharose beads, and

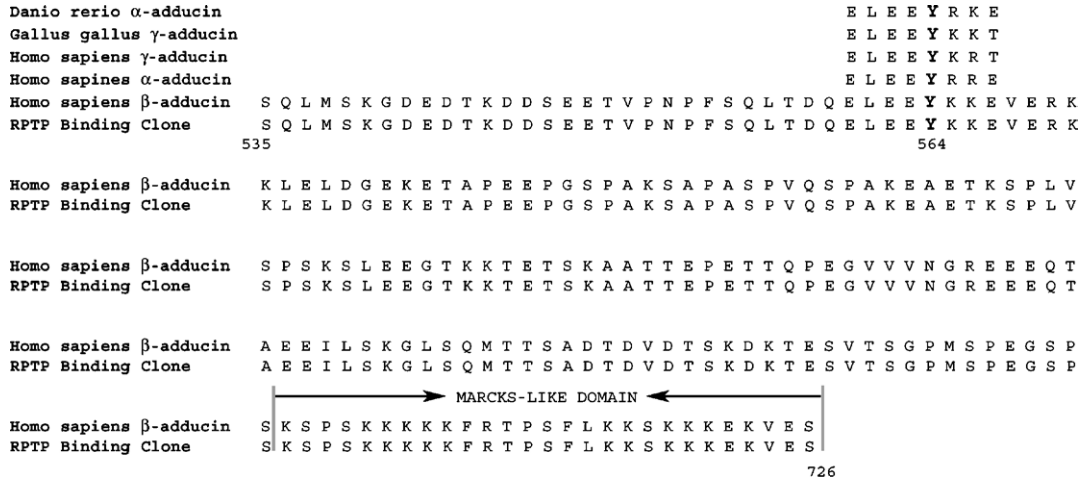

Fig. 3. Identification of the 191 C-terminal amino acid sequence of β-adducin. Amino acid sequence of the RPTPβ/ζ interactive isolated β-adducin clone (amino acid 535–726) and the α- and γ-adducin isoforms. Residue 564 is the putative tyrosine phosphorylation site in β-adducin and highly conserved in α- and γ-adducin as well as *D. rerio* and *G. gallus* adducins. The MARCKS domain in β-adducin (**KSPSKKKKKFRTPSFLKKSKKKEKVES**), (residue 700–726), derived from the similar motif in the MARCKS protein is highlighted.

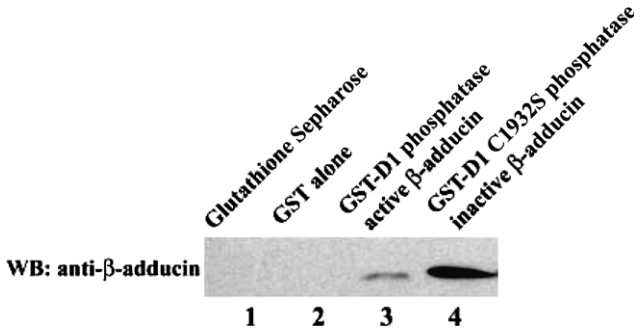

Fig. 4. RPTPβ/ζ D1 domain of capture. Lysates from HeLa cells were incubated with glutathione–Sepharose alone (lane 1), GST alone (lane 2), GST-D1 domain of RPTPβ/ζ (lane 3) and GST-D1 (C1932S) domain of RPTPβ/ζ (lane 4). The GST-coupled proteins "captured" from the lysates were probed with an anti-β-adducin antibody in Western blots.

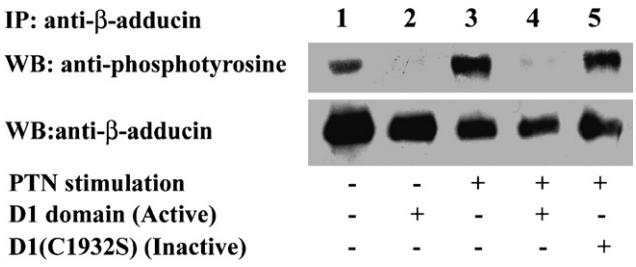

Fig. 5. Dephosphorylation of β-adducin from PTN-stimulated HeLa cells by the D1 domain of RPTPβ/ζ. Lysates from HeLa cells not stimulated (lane 1 and 2) and stimulated with PTN (lane 3–5) were immunoprecipitated with anti-β-adducin antibodies and incubated with the RPTPβ/ζ D1 phosphatase active (lanes 2 and 4) or RPTPβ/ζ D1 (C1932S) (lane 5) phosphatase inactive domain of RPTPβ/ζ and analyzed in Western blots probed with an anti-phosphotyrosine antibodies and re-probed with anti-β-adducin antibodies. Lane 1, β-adducin is phosphorylated in tyrosine in HeLa cells not stimulated with PTN, lane 2, β-adducin in HeLa cells not stimulated with PTN is dephosphorylated by RPTPβ/ζ D1, lane 3, The steady-state levels of tyrosine phosphorylation of β-adducin are increased in PTN-stimulated HeLa cells. Lane 4, β-adducin phosphorylated in tyrosine is dephosphorylated by the phosphatase activity of RPTPβ/ζ D1. Lane 5, β-adducin phosphorylated in tyrosine is not dephosphorylated by the inactivated RPTPβ/ζ D1 (C1932S).

incubated with lysates of confluent HeLa cells. Proteins "captured" by GST-RPTPβ/ζ D1 and GST-RPTPβ/ζ D1 (C1932S) were eluted with 1 mM glutathione and analyzed in Western blots probed with anti-β-adducin- specific antibodies. Both GST-RPTPβ/ζ D1 and GST-RPTPβ/ζ D1 (C1932S) captured a protein recognized in Western blots as β-adducin (Fig. 4, lanes 3 and 4) but GST alone coupled to glutathione–Sepharose (as control) did not, demonstrating that β-adducin interacts with the active site containing D1 domain of RPTPβ/ζ in GST-capture assays.

GST-RPTPβ/ζ D1 (C1932S) captured significantly more β-adducin from the same cell lysates than was captured by GST-RPTPβ/ζ D1 (Fig. 4, lanes 3 and 4), suggesting that in these lysates, β-adducin is phosphorylated in tyrosine and dephosphorylated during incubation by GST-RPTPβ/ζ D1. In this case, β-adducin dephosphorylated in tyrosine, the product of GST-RPTPβ/ζ D1, is anticipated to have a major loss of affinity for GST-RPTPβ/ζ D1, resulting in its capture significantly less efficiently by GST-RPTPβ/ζ D1 than by GST-RPTPβ/ζ D1 (C1932S).

This result thus not only demonstrated the association of β-adducin with the active site domain of RPTPβ/ζ, but supported the possibilities that β-adducin is phosphorylated in tyrosine in lysates of HeLa cells and a substrate of RPTPβ/ζ.

Pleiotrophin increases the steady-state levels of tyrosine phosphorylation of β-adducin in PTN-stimulated cells β-adducin is a substrate of RPTPβ/ζ

To test the possibility that β-adducin is a target of the PTN/RPTPβ/ζ signaling pathway, lysates were prepared from control HeLa cells (Fig. 5, lanes 1 and 2) and from HeLa cells that were stimulated with 50 ng/ml PTN for 30 min (Fig. 5, lanes 3–5). The lysates were immunoprecipitated with anti-β-adducin antibodies, and the immunoprecipitates were solubilized and analyzed in Western blots probed with anti-phosphotyrosine antibodies. To confirm the identity of the phosphoproteins identified with anti-phosphotyrosine antibodies as β-adducin, the blots were then stripped and reprobed with anti-β-adducin-specific antibodies. It was found that β-adducin is phosphorylated in tyrosine in non-PTN-stimulated HeLa cells (Fig. 5, lane 1) as suggested above and that the levels of tyrosine phosphorylation of β-adducin are sharply increased in PTN-stimulated HeLa cells (Fig. 5, lanes 3 and 5). When lysates prepared from both PTN-stimulated and non-PTN-stimulated HeLa cells were incubated with either GST-RPTPβ/ζ D1 or GST-RPTPβ/ζ D1 (C1932S), the levels of β-adducin phosphorylated in tyrosine in lysates from both PTN-stimulated and non-PTN-stimulated cells were markedly reduced when incubated with GST-RPTPβ/ζ D1 (Fig. 5, lanes 2 and 4) but were not reduced when incubated with the activate site inactivated GST-RPTPβ/ζ D1(C1932S) (Fig. 5, lane 5), demonstrating directly that PTN sharply increases tyrosine phosphorylation of β-adducin in PTN-stimulated cells, that β-adducin is phosphorylated in tyrosine in both PTN-stimulated and non-stimulated cells, and that β-adducin phosphorylated in tyrosine is a substrate of RPTPβ/ζ D1, but is not dephosphorylated by RPTPβ/ζ D1 (C1932S).

Discussion

The data presented here demonstrate that β-adducin is a substrate of RPTPβ/ζ and a downstream target of

the PTN/RPTPβ/ζ signaling pathway. The data demonstrate that PTN regulates β-adducin through its ability to regulate the steady-state levels of tyrosine phosphorylation of β-adducin and that exogenous PTN stimulates a marked increase in the levels of tyrosine phosphorylation of β-adducin. The data suggest that PTN regulates β-adducin and the previously identified RPTPβ/ζ substrate β-catenin in concert in PTN-stimulated cells and thus PTN-stimulated tyrosine phosphorylation of β-adducin may participate coordinately with β-catenin in the PTN-stimulated desestabilization of cytoskeleton, increased plasticity, and loss of homophilic cell–cell adhesion that is characteristic of PTN-stimulated cells. The data furthermore are consistent with the conclusion that the endogenous PTN/RPTPβ/ζ signaling pathway functions to maintain the equilibrium of phosphorylated and non-phosphorylated levels β-adducin and β-catenin in different cellular contexts and thus to "fine tune" cytoskeletal structure and fluidity through this mechanism.

β-adducin is known to be an important cytoskeletal protein; it forms hetero-tetramers with α-adducin which associate with the growing ends of actin filaments and with spectrin/actin junction complexes [45,48] and thus to stabilize actin–spectrin junctions near the cell membrane [43–47]. The adducin isoforms comprise N-terminal globular head domains (39 kDa) that are important in the formation of the αβ-adducin hetero-tetrameric complex, small neck domains (9 kDa), and protease sensitive tail domains that directly bind to both actin and spectrin [45,49]. The adducin isoforms share a C-terminal myristoylated alanine-rich protein kinase C substrate (MARCKS)-like domain that contains the important functional protein kinase C (PKC) phosphorylation sites at serines 713 and 726. When serines 713 and 726 are phosphorylated, the affinity of β-adducin with actin and spectrin is sharply reduced, leading to disruption of the spectrin/actin/adducin membrane-associated network, cytoskeletal remodeling, and increased cytoskeletal plasticity.

A recent report [50] demonstrated that β-adducin transiently co-expressed together with the Src family member Fyn is phosphorylated and recruited to the plasma membrane. It was suggested that tyrosine phosphorylation of β-adducin decreases the pool of available β-adducin necessary to directly stabilize the actin cytoskeleton, thereby permitting cells to change shape or to initiate migration. These results [50] thus support the hypothesis that the PTN-stimulated increase in tyrosine phosphorylation of β-adducin is important and it is therefore possible that PTN-stimulated tyrosine phosphorylation of β-adducin may contribute to desestabilization of cytoskeletal complexes and cytoskeletal plasticity through the initiation of migration of β-adducin in PTN-stimulated cells. These findings also raise the possibility that Fyn is the tyrosine kinase whose activity increases the levels of tyrosine phosphorylation of β-adducin in PTN-stimulated cells.

Recently, GIT1/Cat-1 also was identified as a substrate of RPTPβ/ζ using a yeast substrate trapping system [51], expanding the tyrosine phosphorylated proteins that are substrates of RPTPβ/ζ and thus downstream targets of the PTN/RPTPβ/ζ signaling pathway. Overexpression of GIT1 in HEK293 cells inhibits G protein-coupled receptor internalization [52,53] and tyrosine phosphorylation of GIT1 is increased following cell spreading on fibronectin. The levels of tyrosine phosphorylation of GIT1 are regulated during the cell cycle [54], potentially also expanding the cellular regulatory systems regulated by PTN. When we searched different data bases, similarities in potential phosphorylation sites in GIT1/Cat-1 with β-catenin and β-adducin were not found, supporting the hypothesis that RPTPβ/ζ substrate specificity is promiscuous. The promiscuity of substrate specificity suggests the likelihood that a hierarchy of many RPTPβ/ζ substrate proteins is regulated by tyrosine phosphorylation through the PTN/RPTPβ/ζ signaling cascade, and the degree of their regulation is dependent on their relative affinity for RPTPβ/ζ and their relative abundance.

In summary, this work has uncovered β-adducin as a novel downstream target in the PTN-signaling pathway. It was found that PTN regulates the steady-state levels of tyrosine phosphorylation of β-adducin and, suggested that, through regulation of tyrosine phosphorylation levels of β-adducin, PTN may coordinate the functional activities of β-adducin with β-catenin to regulate cytoskeletal remodeling.

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