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# BCCIP Associates With the Receptor Protein Tyrosine Phosphatase PTPμ

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## ABSTRACT

The receptor protein tyrosine phosphatase PTPμ belongs to a family of adhesion molecules that contain cell–cell adhesion motifs in their extracellular segments and catalytic domains within their intracellular segments. The ability of PTPμ both to mediate adhesion and exhibit enzymatic activity makes PTPμ an excellent candidate to transduce signals in response to cell–cell adhesion. In an effort to identify downstream signaling partners of PTPμ, we performed a modified yeast two-hybrid screen using the first tyrosine phosphatase domain of PTPμ as bait. We isolated an interacting clone encoding BRCA2 and CDKN1A interacting protein (BCCIP) from a HeLa cell library. BCCIP is a p21 and BRCA2 interacting protein that has been shown to play roles in both cell cycle arrest and DNA repair. In this manuscript, we confirm the interaction between BCCIP and PTPμ identified in yeast using in vitro biochemical studies and characterize BCCIP as a PTPμ binding protein. We demonstrate that BCCIP is phosphorylated by the Src tyrosine kinase and dephosphorylated by the PTPμ tyrosine phosphatase in vitro. Furthermore, we show that BCCIP is required for both the permissive and repulsive functions of PTPμ in neurite outgrowth assays, suggesting BCCIP and PTPμ are in a common signal transduction pathway. J. Cell. Biochem. 105: 1059–1072, 2008. 

**KEY WORDS:** RECEPTOR PROTEIN TYROSINE PHOSPHATASE; NEURITE OUTGROWTH; BCCIP; NUCLEAR LOCALIZATION

Protein tyrosine phosphorylation is recognized as an important mode of regulation for many cellular functions. The tyrosine phosphorylation state of cellular proteins is determined by the coordinated activity of protein tyrosine kinases (PTKs) [Pawson, 2002; Machida et al., 2003] and protein tyrosine phosphatases (PTPs) [Tonks, 2006]. PTPs are a diverse group of enzymes that exist as either soluble cytoplasmic forms or as transmembrane, receptor-like proteins [Tonks, 2006]. In this manuscript, we focus on the receptor protein tyrosine phosphatase (RPTP) PTPμ. The extracellular segment of PTPμ contains sequences found in several cell adhesion molecules including a Meprin-A5-Mu (MAM) domain, an Ig domain and four FNIII (fibronectin III) repeats. As suggested by the presence of these domains, PTPμ mediates cell–cell adhesion via homophilic binding [Brady-Kalnay et al., 1993; Gebbink et al., 1993b] meaning the "ligand" for PTPμ is an identical PTPμ molecule on an adjacent cell. The juxtamembrane domain (JMD) of PTPμ contains a region homologous to the conserved intracellular domain of the cadherins. Cadherins are calcium-dependent cell–cell adhesion molecules that associate with the actin cytoskeleton via catenins [Gumbiner, 2005; Lilien and Balsamlo, 2005]. Members of the catenin family bind the intracellular domain of cadherins and include α-catenin, β-catenin, plakoglobin, and p120. PTPμ associates with cadherin–catenin complexes [Brady-Kalnay et al., 1995, 1998; Hiscox and Jiang, 1998; Sui et al., 2005]. Specifically, PTPμ interacts with classical cadherins such as N-cadherin, E-cadherin, and R-cadherin [Brady-Kalnay et al., 1998] and VE-cadherin [Sui et al., 2005]. PTPμ has been shown also to interact with p120 catenin [Zondag et al., 2000]. The intracellular domain of PTPμ contains two conserved catalytic domains. Of these two phosphatase domains, only the membrane proximal domain possesses catalytic activity [Gebbink et al., 1993a].

Abbreviations used: PTP, protein tyrosine phosphatase; RPTP, receptor protein tyrosine phosphatase; MAM, Meprin-A5-Mu; FNIII, fibronectin III; GST, glutathione *S*-transferase; CDK, cyclin-dependent kinase; TOK-1, twenty-one and CDK associated protein 1; BCCIP, BRCA2 and CDKN1A interacting protein; RGC, retinal ganglion cell.

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PTPμ has been implicated previously in the regulation of E-cadherin-mediated cell–cell adhesion [Hellberg et al., 2002] and both N-cadherin and E-cadherin-mediated neurite outgrowth [Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007]. Furthermore, PTPμ has been shown to interact directly with IQGAP1 [Phillips-Mason et al., 2006], a protein involved in the regulation of cadherin-mediated cell–cell adhesion [Kuroda et al., 1998; Li et al., 1999]. In addition to PTPμ's role in the regulation of cadherin function, PTPμ appears to either directly or indirectly regulate protein kinase C. In support of this, PTPμ was found to interact with the scaffolding protein RACK1 (receptor for activated C-kinase) in a yeast two-hybrid screen [Mourton et al., 2001], and both PTPμ-mediated neurite outgrowth and PTPμ-mediated axon guidance of retinal ganglion cells (RGCs) require PKCδ activity [Rosdahl et al., 2002; Ensslen and Brady-Kalnay, 2004].

In an effort to elucidate PTPμ-mediated signaling events, we set out to identify PTPμ-interacting proteins using a modified yeast two-hybrid screen in which we co-expressed PTPμ with a constitutively active Src tyrosine kinase. The presence of active Src increased the potential to identify proteins that interact in response to tyrosine phosphorylation. In this manuscript, we describe BCCIP as a PTPμ-interacting protein isolated by this modified two-hybrid screen. BCCIP was originally described as TOK-1 (twenty-one and CDK associated protein-1) based on its ability to bind the cyclin-dependent kinase (CDK)- inhibitor, $p21^{Waf1/Cip1}$ (referred to as p21 hereafter) in a cyclin/CDK2/p21 ternary complex and to enhance the inhibitory activity of p21 on CDK2 [Ono et al., 2000]. Independently, a second group identified BCCIP as a protein that interacts with the internal conserved region of BRCA2, and showed that overexpression of BCCIP inhibits the growth of some tumor cell lines [Liu et al., 2001]. TOK-1 and BCCIP are identical proteins existing as two isoforms in humans, BCCIPα and BCCIPβ [Ono et al., 2000; Liu et al., 2001]. In addition to interacting with p21, BCCIP has been shown recently to regulate p21 expression in a p53-dependent manner [Meng et al., 2004b]. In lower organisms, including rodents, only one BCCIP isoform exists [Lu et al., 2005]. A yeast homolog of BCCIP, Bcp1 has been identified and demonstrated to function in the nuclear export of a protein involved in the regulation of the yeast PI4P 5-kinase signaling molecule, Mss4 [Audhya and Emr, 2003]. In this manuscript, we demonstrate a direct interaction between PTPμ and BCCIP. Furthermore, we show BCCIPα is tyrosine phosphorylated in vitro and a substrate for PTPμ. The best characterized biological function of PTPμ is neurite outgrowth. The catalytic activity of PTPμ is required for it to promote neurite outgrowth of nasal RGCs and induce repulsion of temporal RGCs [Ensslen-Craig and Brady-Kalnay, 2005]. In this manuscript, we demonstrate that expression of a BCCIP antisense construct blocks both permissive and repulsive activities of PTPμ in neurite outgrowth assays of RGCs, suggesting BCCIP and PTPμ are in a common signaling pathway.

## MATERIALS AND METHODS

### ANTIBODIES

A monoclonal antibody generated against the intracellular domain of PTPμ (SK18) has been described previously [Brady-Kalnay and Tonks, 1993; Brady-Kalnay and Tonks, 1994] and was used to detect PTPμ. An HRP-conjugated antibody to T7 (Novagen, Madison, WI) was used to detect the T7 tag in His-iPTPμ constructs. Antibodies to BCCIPα,β (TOK-1α,β) and Lamin A/C were purchased from Transduction Laboratories (San Diego, CA). An antibody that recognizes vinculin was purchased from Sigma Chemical Company (St. Louis, MO) and a monoclonal antibody to phosphotyrosine (PY99) was obtained from Santa Cruz (Santa Cruz, CA).

### CELL CULTURE

Sf9 insect cells (CRL 1711: American Type Tissue Culture, Manassas, VA) were maintained at 27°C in Grace's Insect Medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum and 1 μg/ml gentamicin. A549 human non-small cell lung carcinoma cells (CCL 185: American Type Tissue Culture) were maintained in F12 media (Invitrogen Corp.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1 μg/ml gentamicin at 37°C, 5% $CO_2$. LNCaP human prostate cancer cells were maintained in RPMI media (Invitrogen Corp.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1 μg/ml gentamicin at 37°C, 5% $CO_2$. PrEC, normal human prostate epithelial cells (BioWhittaker, Walkersville, MD) were maintained at 37°C, 5% $CO_2$ in PrEGM growth media (BioWhittaker).

E6 retinal cell cultures were prepared as described previously [Burden-Gulley and Brady-Kalnay, 1999]. Briefly, E6 (stage 28) chick neural retinas were dissociated in 0.25% trypsin, 4Na EDTA (Invitrogen Corp.) for 20 min, followed by mechanical trituration. Cells were resuspended and cultured in 10% fetal bovine serum (Hyclone), 2% chick serum (Sigma, St. Louis, MO), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.025 μg/ml amphotericin in RPMI (Invitrogen Corp.).

### MODIFIED YEAST TWO-HYBRID SCREEN

We utilized an Interaction Trap/Two-Hybrid system to identify PTPμ interacting proteins originally described by Erica Golemis [Golemis, 1997; Mourton et al., 2001]. There are four essential components of the system: the bait (PTPμ), the prey (potential interacting proteins from a library) and two reporters (*LEU2* and *lacZ*) that allow detection of the interaction between the bait and prey. Briefly, a construct containing the membrane-proximal catalytic domain of human PTPμ (PTPμD1, amino acids 915–1178) was cloned into pEG202 (*HIS3*) to yield pEG202-D1, and this plasmid was used as bait. The pEG202 plasmid constitutively expresses a chimeric protein containing the DNA-binding domain of LexA fused to the bait. In the absence of an interacting library protein, the LexA-bait fusion is unable to activate either of the two reporters. pEG202-D1 and the β-galactosidase reporter plasmid, pSH18-34 (*URA3*), were co-transformed into the YPH499 yeast strain. The reporter plasmid pSH18-34 carries a LexA operator-*lacZ* fusion gene. A conditionally expressed HeLa cell cDNA library in the pJG4-5 (*TRP1*) yeast expression vector was introduced into a yeast strain containing a chromosomal copy of the *LEU2* gene (EGY48). The upstream activating sequences for the chromosomal *LEU2* gene in the EGY48 strain were replaced with LexA operators. *LEU2* is required for biosynthesis of leucine. The library proteins are expressed, under an inducible yeast GAL1 promoter, as fusions to an acid blob that

function as a transcriptional activation motif. Library protein expression is induced by plating transformants on medium containing galactose. There is no expression of library proteins in the presence of glucose, which is used as a negative control. The two yeast strains, EGY48 and YPH499, were mated. Potential interactions were detected by growing the mated yeast strain on minimal media containing 2% galactose and 1% raffinose lacking certain amino acids to ensure selective pressure. The two reporters allow selection for transcriptional activation by permitting selection for viability when cells are plated on media lacking leucine and selected based upon blue color when the yeast are grown in Xgal-containing media. Library proteins that do not interact specifically with the bait fail to grow in the absence of Leu. Yeast that contain PTPμ-interacting proteins will turn blue only when plated in the presence of Xgal and galactose.

In the modified yeast two-hybrid screen, the constitutively active form of the non-receptor protein tyrosine kinase *src* provided by Dr. Jonathan Cooper [Keegan and Cooper, 1996] was ligated into the pSH18-34 plasmid. The pSH18-34/*src* and pEG202-D1 plasmids were used to transform the YPH499 yeast strain. This transformed yeast strain was then mated to the EGY48 strain containing the HeLa cell cDNA library to isolate interacting clones that are dependent upon or enhanced by tyrosine kinase activity.

### EXPRESSION OF FUSION PROTEINS IN *E. coli*

Plasmids containing glutathione *S*-transferase (GST) fusion proteins of full length BCCIPα (pGEX-GST-TOK-1α) and BCCIPβ (pGEX-GST-TOK-1β) were kindly provided by Dr. Hiroyoshi Ariga (Hokkaido University, Japan). GST-BCCIPα, GST-BCCIPβ and GST alone were expressed in *E. coli* under the control of the *lac* promoter. The BCCIP fusion proteins and GST were purified from *E. coli* using glutathione Sepharose 4B beads (Amersham Biosciences) and used in the GST pull-down experiments described below. Plasmids containing intracellular GST-PTPμ fusion proteins (GST-iPTPμ:765-1452, GST-iPTPμ:765-1178 and GST-PTPμ:765-958) were described previously [Phillips-Mason et al., 2006]. These fusion proteins were also expressed in bacteria under the control of the *lac* operon and isolated on glutathione Sepharose as described above.

The pTrcHis plasmid (Invitrogen Corp.) is used to generate T7, 6(His)-tagged fusion proteins in bacteria. Using PCR, we generated a plasmid encoding the JMD, and first phosphatase domain of PTPμ (His-iPTPμ:774–1161) that expresses a T7/His-tagged fusion protein containing amino acids 774–1161 of PTPμ. Similarly, a plasmid encoding the second phosphatase domain of PTPμ (His-iPTPμ:1213–1452) that expresses a T7/His-tagged fusion protein containing amino acids 1213–1452 was generated. Finally, a plasmid that expresses a T7/His-tagged fusion protein containing amino acids 915–1161 of PTPμ was generated (His-iPTPμ:915–1161). This construct is similar to the yeast two-hybrid bait (915–1161 vs. 915–1178). The T7/His-tagged fusion proteins were expressed in *E. coli* under the control of the *lac* operon. His-iPTPμ:915–1161 was purified using the PrepEase™ His-Tagged Protein Purification Kit (USB Corporation, Cleveland, OH).

### BACULOVIRUS EXPRESSION

A baculovirus encoding BCCIPα was generated by digesting the pGEX-GST-TOK-1α plasmid with *Bam*HI and *Not*I restriction enzymes and ligating the TOK-1/BCCIP insert into pVL1392 (Pharmingen) linearized with *Bgl*II and *Not*I restriction enzymes. Baculovirus was generated using the BCCIP/pVL1392 plasmid and the BaculoGold™ system (BD Pharmigen). Sf9 cells were infected with virus stocks, and 48 h post-infection, cells were harvested in a lysis buffer containing 20 mM Tris, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM benzamidine, 0.2 mM phenyl arsine oxide, 1 mM sodium orthovanadate, 0.1 mM molybdate, and protease inhibitor cocktail (Sigma Chemical Company), and used in GST pull-down experiments as described below.

### GST PULL-DOWN EXPERIMENTS

Ten micrograms of *E. coli* protein lysate from cells expressing the His-iPTPμ:774-1161 or His-iPTPμ:1213-1452 constructs were incubated with glutathione Sepharose beads which had been bound previously to GST-BCCIPα, GST-BCCIPβ or GST alone in lysis buffer containing 20 mM Tris, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM benzamidine, 0.2 mM phenyl arsine oxide, 1mM sodium orthovanadate, 0.1 mM molybdate, and protease inhibitor cocktail. Samples were rocked overnight at 4°C. The next day, samples were washed four times in lysis buffer and incubated at 95°C for 5 min in 2× SDS sample buffer. One fifth of the sample was resolved by SDS–PAGE and transferred to nitrocellulose for immunoblot analysis as described previously [Brady-Kalnay et al., 1995]. A similar experiment was done using 50 μg of protein lysate from Sf9 cells expressing BCCIPα incubated with GST-PTPμ fusion proteins immobilized on glutathione Sepharose. Pull-downs were also done using 500 μg of protein lysate from A549 cells treated with 100 μM pervanadate for 20 min, or untreated. These lysates were incubated with GST, GST-BCCIPα or GST-BCCIPβ immobilized on glutathione Sepharose in lysis buffer as described above.

### IN VITRO SRC KINASE ASSAY

GST-BCCIPα and GST-BCCIPβ fusion proteins and GST alone were expressed and purified from bacteria as described above. Thirty micrograms of each fusion protein was used in an in vitro Src kinase reaction. Five-hundred microlitres of kinase buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 0.1 mM NaV, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 5 mM ATP) was added to 30 μg of fusion protein along with 5 μl (15U) of purified Src tyrosine kinase (Catalog number 14-117, Upstate, Lake Placid, NY) or buffer, and the samples were incubated at room temperature for 2 h. Samples were separated by SDS–PAGE and transferred to nitrocellulose. Tyrosine phosphorylation was detected by immunoblot with an anti-phosphotyrosine antibody. These fusion proteins were subsequently used in pull-down experiments with purified His-iPTPμ:915–1161.

### PTPμ PHOSPHATASE ASSAY

GST-BCCIPα was phosphorylated in vitro by Src, as described above. After the Src kinase reaction, the phosphorylated BCCIPα sample was divided into three samples. One sample was kept as a control for the kinase reaction, and the remaining two were washed three times in phosphatase buffer (25 mM Hepes, pH 7.4, 50 mM

NaCl and 5 mM DTT). Phosphatase buffer (500 μl) was added to the samples, and active GST-iPTPμ:765–1178 isolated as previously described [Phillips-Mason et al., 2006] was added to one sample. Samples were incubated at 37°C for 15 min and then stopped by the addition of 2× sample buffer. Samples were separated by SDS–PAGE and transferred to nitrocellulose membrane. Tyrosine phosphorylation was analyzed by immunoblot with an anti-phosphotyrosine antibody.

### IMMUNOCYTOCHEMISTRY

A549, PrEC, and LNCaP cells were grown on Lab Tech II chamber slides (Nalge Nunc International Corp., Rochester, NY) for 1–2 days. Cells were fixed with 4% paraformaldehyde for 10 min. Cells were then washed with PBS three times and permeabilized using 0.5% saponin in blocking buffer (20% goat serum, 1% BSA in PBS) for 30 min. Primary antibodies were diluted in blocking buffer plus 0.5% saponin and incubated overnight at 4°C. After incubation with primary antibody, cells were rinsed five times with TNT buffer (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20). Secondary antibodies (either goat anti-mouse-FITC or goat anti-rabbit-FITC from ICN Biochemicals, Irvine, CA) were diluted in blocking buffer plus saponin and incubated for 1 h at room temperature. After incubation with secondary antibody, cells were washed five times with TNT buffer and once with distilled water. Molecular Probes SlowFade® Light Antifade kit was used to minimize quenching. Slides were imaged using a Nikon (Tokyo, Japan) TE200 inverted microscope and images were collected with a Spot RT digital camera and image acquisition software (Diagnostic Instruments, Inc., Sterling Heights, MI).

### SUBCELLULAR FRACTIONATION

A549, PrEc, and LNCaP cells were grown in 10 cm dishes to 80–95% confluence. Cells were washed twice with PBS, and 500 μl of cold lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, and 10% (v/v) glycerol) was added to the cells. Cells were collected by gentle scraping into a microfuge tube, incubated on ice for 10 min and then dounced fifty times on ice with a size B pestle. Dounced cells were centrifuged at 3,000*g* for 10 min at 4°C to pellet nuclei. The supernatant was collected and centrifuged at 100,000*g* for 60 min at 4°C; the supernatant of this spin was designated as cytosol. Nuclear pellets were resuspended in 200 μl cold lysis buffer and layered over an equal volume of 50% sucrose (w/v) in lysis buffer and centrifuged at 14,000*g* for 15 min at 4°C. Nuclear pellets were resuspended in cold lysis buffer with 0.1% Triton X-100, briefly sonicated and insoluble material pelleted by centrifuging at 14,000*g* for 4 min at 4°C. Protein concentration of the cytosolic and nuclear lyastes was determined by the Bradford method [Bradford, 1976], using BSA as standard. Lysates were incubated with equal amounts of 2× sample buffer at 95°C for 5 min. Twenty micrograms of both cytosolic and nuclear proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes for analysis by immunoblotting with various antibodies.

### LENTIVIRUS PREPARATION AND CELL INFECTION

A human full-length PTPμ-GFP construct in pBPSTR1 has been described previously [Burden-Gulley and Brady-Kalnay, 1999]. We constructed a PTPμ-GFP lentiviral construct by inserting the *Not*I fragment from PTPμ-GFP/pBPSTR1 into the lentiviral expression vector pCDH-MCS2 (System Biosciences, Mountain View, California). An intracellular PTPμ-765-1451-GFP construct was generated using PCR. The PCR fragment was inserted into pCDH-MCS2 using the *Xba*I and *Not*I restriction sites in the MCS. VSV-G-pseudotyped lentiviral particles were generated by triple transfection of pCDH-MCS-2 constructs with the packaging plasmids pCMVΔR8.91 and pMD.G into 293T cells using Lipofectamine 2000 (Invitrogen Corp.) according to a previously described protocol [Dull et al., 1998]. A549 cells were infected with lentiviral particles concentrated by ultracentrifugation in the presence of 6 μg/ml of polybrene. Immunocytochemistry was performed 24 h post-infection.

### RECOMBINANT HSV PREPARATION AND CELL INFECTION

The antisense PTPμ-HSV plasmid used to generate recombinant HSV was previously described [Ensslen et al., 2003]. To generate an antisense BCCIP–HSV plasmid, the human BCCIPβ insert was excised from BCCIPβ/pGEX-FLAG via digestion with *Bam*HI and *Xho*I restriction enzymes and inserted in the antisense orientation into the pHSVpuc-MCS vector. The virus was produced as described previously [Ensslen et al., 2003] by transfection of the recombinant plasmids into a helper cell line (2–2 Vero cells), followed by infection with the 5d11.1 helper virus. After three amplification cycles, the virus was concentrated by ultracentrifugation and stored at −80°C.

E6 retinal cells and LNCaP cells were infected with 10 μl of HSV in basal media (RPMI and F12, respectively). After 2 h incubation, 2× complete media was added to the cells to give a final concentration of 1×. Cells were incubated for a total of 24 h in the presence of HSV prior to harvesting the cells for protein extraction.

### BONHOEFFER STRIPE ASSAY

The stripe assay used was a modified version of the Bonhoeffer method [Vielmetter et al., 1990] previously described [Burden-Gulley et al., 2002]. Briefly, tissue culture dishes were coated with nitrocellulose [Lagenaur and Lemmon, 1987] and allowed to dry before applying a silicon lane matrix to the culture dish. Alternating stripes of laminin and PTPμ-Fc chimera containing Texas-Red conjugated BSA (for visualization) were generated as follows for single stripe assays. Eighty nanograms of PTPμ-Fc chimera described previously [Rosdahl et al., 2003], was injected into the channels of the silicon lane matrix, incubated for 10 min, aspirated and then replaced with a new aliquot of PTPμ-Fc substrate for two additional 10 min incubations. Any remaining binding sites within the lanes were blocked with bovine serum albumin (BSA; fraction V; Sigma) and rinsed with calcium-magnesium free phosphate buffer (CMF). The matrix was removed and 1.75 μg of laminin (BTI, Stoughton, MA) was spread across the lane area and incubated for 20 min. Explants from E8 chick nasal retina were grown on the alternating stripes of laminin and PTPμ-Fc for 48 h in 10% fetal bovine serum (Hyclone), 2% chick serum (Sigma), 2 mM L-glutamine, 2 U/ml penicillin, 2 μg/ml streptomycin and 5 ng/ml amphotericin in RPMI (Invitrogen Corp.). Quantitation of the stripe assays was performed using a rating scale previously described

[Walter et al., 1987; Ensslen and Brady-Kalnay, 2004]. Neurites that show no preference for either substrate are assessed at zero. Neurites that grow exclusively on one substrate are assessed at three. Neurites that grow mainly on the laminin lanes with an occasional neurite crossing over PTPμ lanes, are assessed at two, while an assessment of one is given when there is a significant amount of neurite crossing but a tendency to fasciculate on laminin. For mixed substrate stripe experiments, 160ng PTPμ-Fc mixed with 0.9 μg laminin containing Texas Red BSA was used as the first substrate as described above. For the second substrate, 1.75 μg of laminin was spread over the lane area. The entire dish was blocked with BSA and rinsed with RPMI. When viral perturbations were used, HSV was added at the time of explant. Statistical significance between the different treatments was determined using an unpaired *t* test.

## RESULTS

### MODIFIED YEAST TWO-HYBRID ANALYSIS

A modified yeast two-hybrid screen was performed to identify PTPμ interacting proteins. In this modified scheme, a constitutively active Src kinase was co-expressed as a strategy for identifying protein-protein interactions that are dependent upon, or strengthened by, tyrosine phosphorylation. This modified yeast two-hybrid scheme has been used previously to identify protein interactions that are enhanced by phosphorylation [Keegan and Cooper, 1996; Lioubin et al., 1996]. Yeast containing either the first phosphatase domain of PTPμ as bait or the first phosphatase domain of PTPμ and constitutively active Src were mated with yeast containing a HeLa cell cDNA library. Only yeast containing both bait and prey plasmids will grow on galactose, and the β-galactosidase reporter will be activated only when interaction of the bait and a prey protein occurs. There is no expression of library proteins when the yeast are plated on glucose. Using this system, we isolated a prey construct encoding full length BCCIPα from the HeLa cell library. PTPμ and BCCIPα interacted in yeast on galactose only in the presence of constitutively active Src as indicated by β-galactosidase staining of the yeast colony (Fig. 1A). These data suggest the interaction between BCCIP and PTPμ is directly or indirectly stabilized by Src kinase activity in the yeast system. BCCIP is a p21 [Ono et al., 2000; Meng et al., 2004b] and BRCA2 [Liu et al., 2001; Lu et al., 2005] interacting protein. In human cells, two BCCIP isoforms, BCCIPα and BCCIPβ, are expressed as the result of alternative splicing [Ono et al., 2000; Liu et al., 2001]. The isoforms are identical through the first 258 amino acids but have unique C-termini. BCCIPα has a 64 amino

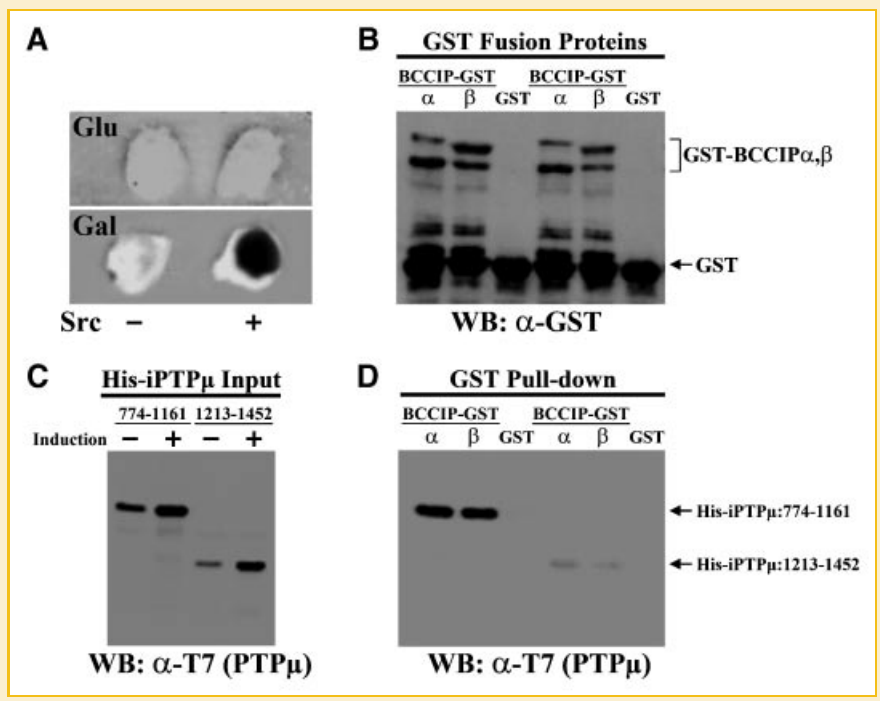


Fig. 1. PTPμ and BCCIPα interact in a modified yeast two-hybrid screen and in vitro. A: The final results of the modified yeast two-hybrid screen are shown. Yeast containing PTPμ and BCCIPα were grown on medium containing either galactose or glucose. Galactose induces the expression of the prey protein (BCCIPα) in the yeast. When grown on Xgal-containing media, the PTPμ/BCCIPα strain turns blue on galactose only in the presence of the Src tyrosine kinase. B: GST, GST-BCCIPα and GST-BCCIPβ fusion proteins were isolated on glutathione beads as described in Materials and Methods Section. These GST fusion proteins were used in the pull-down assay described below. C: Bacterial lysates from cells expressing T7-tagged His-iPTPμ:774-1161 and His-iPTPμ:1213-1452 before (−) and after (+) induction with IPTG were separated by SDS-PAGE and protein expression confirmed by immunoblotting with a T7 antibody. D: Post-induction bacterial lysates were used as the input for GST pull-downs. Bacterial lysates of cells expressing either the first (His-iPTPμ:774-1161) or second (His-iPTPμ:1213-1452) phosphatase domain of PTPμ were incubated with GST-tagged BCCIPα, BCCIPβ or GST alone preloaded on glutathione beads. The ability of His-iPTPμ:774-1161 and His-iPTPμ:1213-1452 to associate with BCCIP was determined by separating the associated proteins by SDS-PAGE followed by immunoblotting with a monoclonal antibody to the T7 tag present in the His-iPTPμ:774-1161 and His-iPTPμ:1213-1452 fusion proteins. To ensure equal amounts of the fusion proteins were used in the pull-down assay, the anti-T7 immunoblot from the GST pull-downs was stripped and immunoblotted with an anti-GST antibody and shown in B.

acid C-terminal domain, while BCCIPβ has a 58 amino acid C-terminus. In lower organisms, including rodents, only one BCCIP isoform exists [Lu et al., 2005]. To date, no isoform-specific functions have been demonstrated for BCCIP.

### BCCIP AND PTPμ INTERACT IN VITRO

The first phosphatase domain of PTPμ (amino acids 915–1178) was used as the bait in our modified yeast two-hybrid screen. To verify that BCCIP was interacting with this domain, lysates from bacteria expressing PTPμ constructs corresponding to the JMD and first phosphatase domain (D1) of PTPμ (His-iPTPμ:774–1161) and the second phosphatase domain (D2) of PTPμ (His-iPTPμ:1213–1452), shown in Figure 1C, were incubated with BCCIP-GST fusion proteins isolated on glutathione Sepharose (Fig. 1B) in a pull-down assay (Fig. 1D). Figure 1D shows BCCIP interacts specifically with the His-iPTPμ:774–1161 protein. Furthermore, both BCCIPα and BCCIPβ are equally efficient at interacting with PTPμ in this system. These results suggest that the PTPμ binding site on BCCIP lies within the N-terminal 258 amino acids that both BCCIP isoforms share. GST was used as a negative control and did not interact with either phosphatase domain of PTPμ (Fig. 1D). Figure 1C shows that approximately equal amounts of the His-iPTPμ:774–1161 and His-iPTPμ:1213–1452 protein were expressed. It should be noted that there is a small amount of His-iPTPμ:1213–1452 protein binding to BCCIPα and β (Fig. 1D), this is most likely due to the fact that there is 33% identity and 53% similarity between the D1 and D2 phosphatase domains of PTPμ.

### BCCIPα IS A SUBSTRATE FOR THE Src TYROSINE KINASE AND THE PTPμ TYROSINE PHOSPHATASE IN VITRO

In order to investigate the role of Src phosphorylation on the PTPμ/BCCIP interaction, we first determined whether BCCIP was a substrate for Src in vitro. BCCIPα and BCCIPβ GST fusion proteins isolated on glutathione Sepharose were incubated with Src tyrosine kinase in an in vitro kinase assay. Tyrosine phosphorylation of the BCCIP fusion proteins was determined by immunoblotting with an anti-phosphotyrosine antibody. Figure 2A shows that BCCIPα, but not BCCIPβ is a substrate for Src in vitro. There are no tyrosine residues in the BCCIPα unique region. There are six tyrosine residues in the region common to both BCCIPα and BCCIPβ; three of the six residues (Y64, Y71, and Y257) are predicted to be candidates for phosphorylation by NetPhos 2.0 (www.cbs.dtu.dk/services/netphos). Because both BCCIP isoforms contain all three tyrosine residues that have the potential to be phosphorylated, it is possible that there is some aspect of the unique region of BCCIPβ that prevents phosphorylation, or some aspect of the unique region of BCCIPα that promotes phosphorylation of these residues. Of the three residues, which have phosphorylation potential, perhaps the most interesting is Y257 which resides just one amino acid away from the end of the 258 amino acid sequences that the two BCCIP isoforms share. The fact that only BCCIPα is phosphorylated by Src may explain why this isoform was isolated in the modified yeast two-hybrid screen.

To determine whether tyrosine phosphorylation of BCCIPα altered the ability of BCCIP to interact with PTPμ, pull-downs were performed using the phosphorylated BCCIP fusion proteins and purified His-iPTPμ:915–1161 which encompasses the first phosphatase domain similar to the yeast two hybrid bait (amino acids 915–1161 vs. 915–1178). The bottom panel of Figure 2A (WB: α-PTPμ) shows Src phosphorylation did not have a significant effect on the ability of BCCIPα to bind to PTPμ. Furthermore, BCCIPβ, which is not a substrate for Src tyrosine kinase in vitro, binds PTPμ. Therefore, the Src-dependence of the PTPμ/BCCIP interaction observed in the modified yeast two-hybrid screen, suggests Src is likely to indirectly regulate their interaction in complex systems. These data also suggest that PTPμ and BCCIP interact directly, since

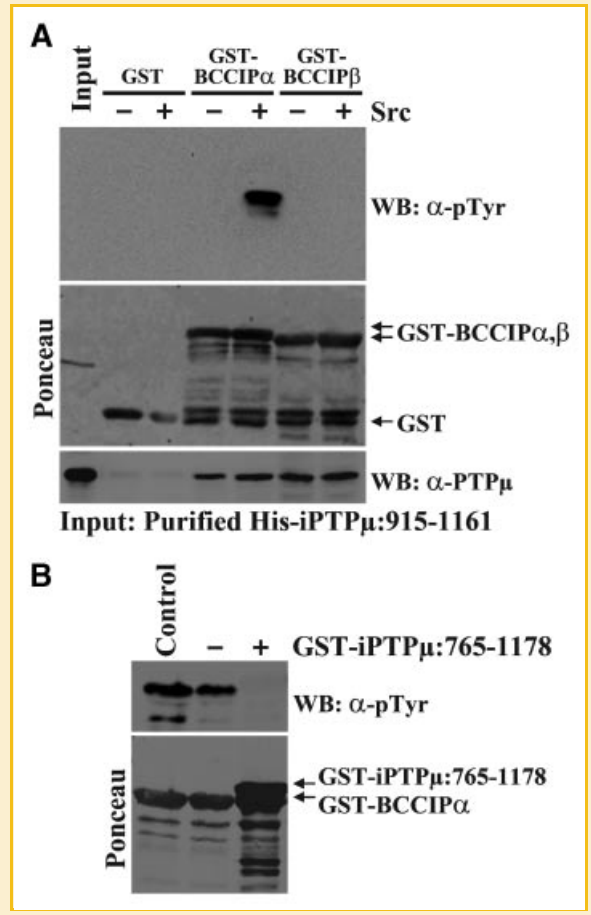


Fig. 2. BCCIPα is an in vitro substrate for both the Src tyrosine kinase and the PTPμ tyrosine phosphatase. A: GST, GST-BCCIPα, and GST-BCCIPβ fusion proteins were isolated on glutathione Sepharose and used in an in vitro Src kinase assay. Following phosphorylation by Src, the GST fusion proteins were used in a pull-down assay with purified His-iPTPμ:915–1161. The pull-down samples were separated by SDS–PAGE, transferred to nitrocellulose and analyzed as follows. The top panel is an immunoblot using an anti-phosphotyrosine antibody and shows BCCIPα is a substrate for Src in vitro. The bottom panel is the same membrane immunoblotted for PTPμ (SK18). The center panel is a Ponceau stain of the membrane showing equal loading of the GST fusion proteins. B: GST-BCCIPα was isolated on glutathione Sepharose and phosphorylated in vitro by Src. After phosphorylation the sample was divided into three samples and either untreated (control), or washed with a phosphatase buffer and incubated with (+) or without (–) GST-iPTPμ:765–1178. The phosphorylation status of BCCIPα was determined by immunoblotting with an anti-phosphotyrosine antibody. The bottom panel is a Ponceau stain of the membrane to show equal loading of GST-BCCIPα.

both the BCCIP and PTPμ used in these experiments were purified proteins.

Since BCCIPα is a substrate for Src in vitro, we were curious to determine whether BCCIPα was a substrate for PTPμ as well. Figure 2B shows that BCCIPα is indeed a substrate for PTPμ in vitro. These data strengthen the hypothesis that the interaction of these proteins is involved in cellular signaling in response to tyrosine phosphorylation.

### THE PTPμ/BCCIP INTERACTION DOMAIN

To characterize further the region of PTPμ that interacts with BCCIP, we performed pull-downs using a series of PTPμ deletion constructs fused to GST (Fig. 3A). Lysates from Sf9 cells expressing BCCIPα were incubated with PTPμ-GST fusion proteins and assayed for their ability to interact with BCCIPα in a pull-down assay. Figure 3B shows that the smallest GST-PTPμ construct tested (GST-iPTPμ:765–958) was able to bind BCCIP. The yeast two-hybrid bait comprises amino acids 915–1178 of PTPμ. Together, these data suggest the minimal overlapping sequence in the PTPμ constructs that interact with BCCIP is amino acids 915–958.

### LOCALIZATION OF ENDOGENOUS BCCIP

PTPμ is a transmembrane protein stabilized at cell–cell contacts [Brady-Kalnay et al., 1995; Gebbink et al., 1995]. BCCIP is a cytosolic protein that contains a potential nuclear localization sequence. Previous studies have shown BCCIP to be predominantly nuclear [Ono et al., 2000; Liu et al., 2001]. Currently, there are only commercial antibodies that recognize the human BCCIP protein effectively. Therefore, we examined the localization of BCCIP in several human cell lines that endogenously express BCCIP (Fig. 4A). Immunocytochemistry was performed using a monoclonal antibody that recognizes both BCCIPα and BCCIPβ. In all three cell lines examined, BCCIP was present in both the cytoplasm and the nucleus, suggesting BCCIP shuttles between the cytoplasm and nucleus. Both A549 human lung cancer cells and PrEC human prostate cells have endogenous PTPμ, but LNCaP human prostate cancer cells have down-regulated PTPμ protein expression by an unknown mechanism [Hellberg et al., 2002]. Figure 4A shows LNCaP and PrEC cells have a similar staining pattern of BCCIP, suggesting the presence of PTPμ protein does not change BCCIP localization in these cells. Adding PTPμ back to LNCaP cells via a lentiviral expression system did not change the localization of BCCIP (data not shown).

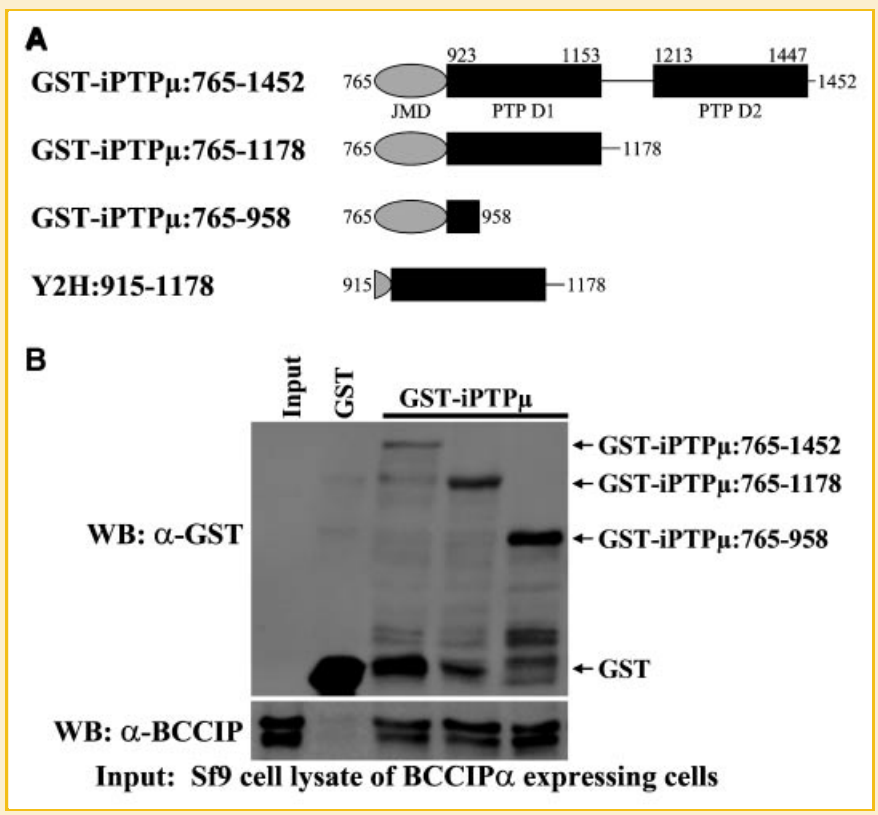


Fig. 3. The PTPμ/BCCIP interaction domain. A: Schematic of intracellular GST-PTPμ constructs used in pull-down experiments. All constructs contain a GST sequence at the N-terminus. A gray oval represents the JMD and black box represents the first (PTP D1) and second (PTP D2) phophatase domains. The yeast two-hybrid bait construct is included for reference. B: GST-iPTPμ constructs and GST alone were immobilized on glutathione Sepharose and incubated with equal amounts of lysate from Sf9 cells expressing BCCIPα. Interaction of BCCIP with the GST-PTPμ fusion proteins was determined by separating associated proteins by SDS–PAGE followed by immunoblotting with a monoclonal antibody to BCCIP. We observe two bands with the monoclonal antibody to BCCIP when we immunoblot lysates from Sf9 cells expressing BCCIPα. This is most likely due to post-translational modification or cleavage. The BCCIP immunoblot was stripped and reprobed with an anti-GST antibody to verify GST fusion protein loading (WB: α-GST).

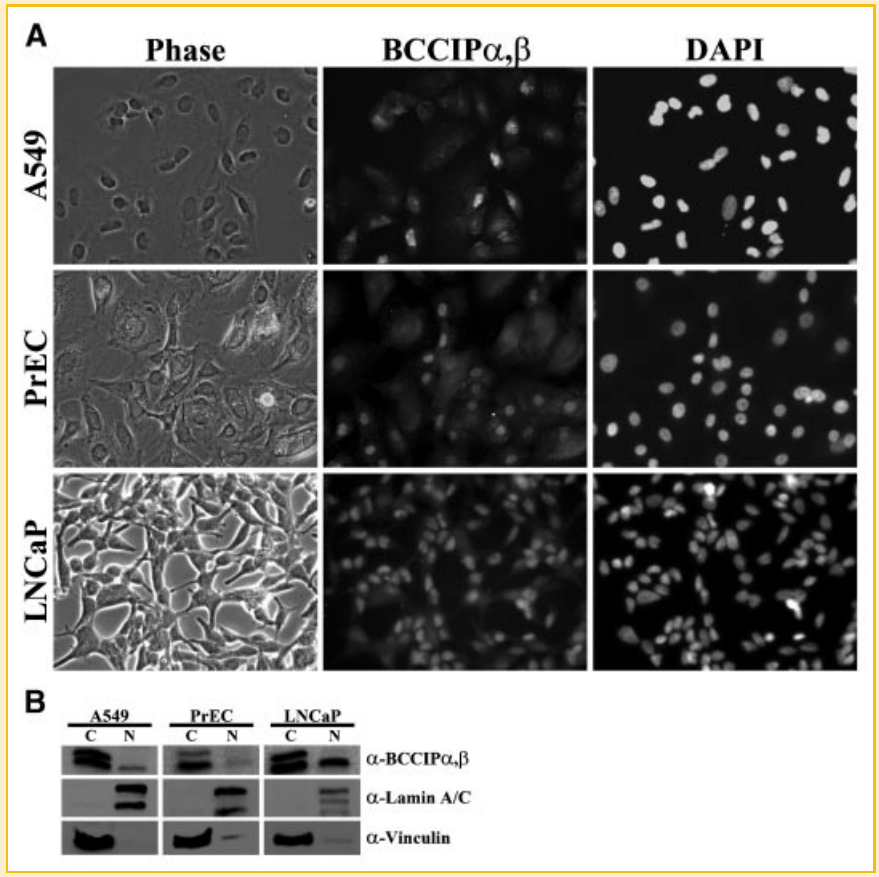


Fig. 4. Localization of endogenous BCCIP. A: Immunocytochemistry was performed on A549, PrEC, and LNCaP cells using a monoclonal antibody that recognizes both BCCIPα and BCCIPβ. In all three cell lines, BCCIP localizes to both the cytoplasm and the nucleus. DAPI staining was included to define the nuclei. B: A549, PrEC, and LNCaP cells were fractionated into nuclear and cytosolic fractions. Isolated fractions were separated by SDS–PAGE and proteins detected by immunoblot using a monoclonal antibody to BCCIP. Purity of the cell fractions was verified using vinculin as a cytosolic (C) marker and lamin A/C as a nuclear (N) marker. There are prominent bands corresponding to BCCIPα and BCCIPβ in the cytosolic fraction (BCCIPα and BCCIPβ are 50 and 45 kDa, respectively). Upon longer exposure, a low level of BCCIPα can be detected in the nuclear fraction.

To verify the localization of BCCIP demonstrtated by immunocytochemistry, we did subcellular fractionation of A549, LNCaP and PrEC cells. Somewhat surprisingly, BCCIPα appeared to be predominately, if not completely, in the cytoplasm, and BCCIPβ was both nuclear and cytosolic (Fig. 4B). These data are consistent with our immunocytochemistry data showing both a cytoplasmic and nuclear pool of BCCIP. The predominant cytoplasmic pool of BCCIPα was unexpected and revealed only by doing cell fractionation and immunoblot analysis, since the BCCIP isoforms cannot be distinguished by immunocytochemistry with the commercially available BCCIPα,β antibody. Our data are not in full agreement with the results of Ono et al. [2000], which demonstrate exogenous BCCIP is a predominately nuclear protein. The source for this discrepancy could be due to the fact that Ono et al. [2000] performed immunocytochemistry on Cos-7 cells overexpressing BCCIP. Consistent with our data, images from Liu et al. [2001] of MCF-7 cells stained for BCCIP, clearly demonstrate endogenous BCCIP is both nuclear and cytoplasmic. We would like to note that we performed immunocytochemistry in A549 cells with a polyclonal antibody specific to BCCIPα [a gift from Dr. Hiroyoshi Ariga (Hokkaido University, Japan), data not shown]. With the BCCIPα-specific antibody, BCCIPα appeared to be mainly cytoplasmic, in agreement with our cell fractionation data. From these data in combination with the cell fractionation data, we concluded that the commercially available antibody directed against both BCCIPα and BCCIPβ, has a higher affinity for native BCCIPβ than native BCCIPα. This may explain why immunocytochemistry performed with the commercial antibody shows a greater nuclear pool than cell-fractionation. Overall, these combined data indicate BCCIPα and BCCIPβ have both cytoplasmic and nuclear localization.

## ENDOGENOUS PTPμ ASSOCIATES WITH BCCIP IN PULL-DOWN ASSAYS

To gain additional evidence that PTPμ and BCCIP interact in vivo, we attempted to co-immunoprecipitate endogenous BCCIP and PTPμ from mammalian cells. Our attempts were unsuccessful, in part, due to the lack of antibodies capable of immunoprecipitating BCCIP. However, we were able to pull-down endogenous PTPμ from A549 cells using BCCIP-GST fusion proteins (Fig. 5A). To evaluate the role of tyrosine phosphorylation in this interaction, A459 cells were treated with or without the tyrosine phosphatase inhibitor

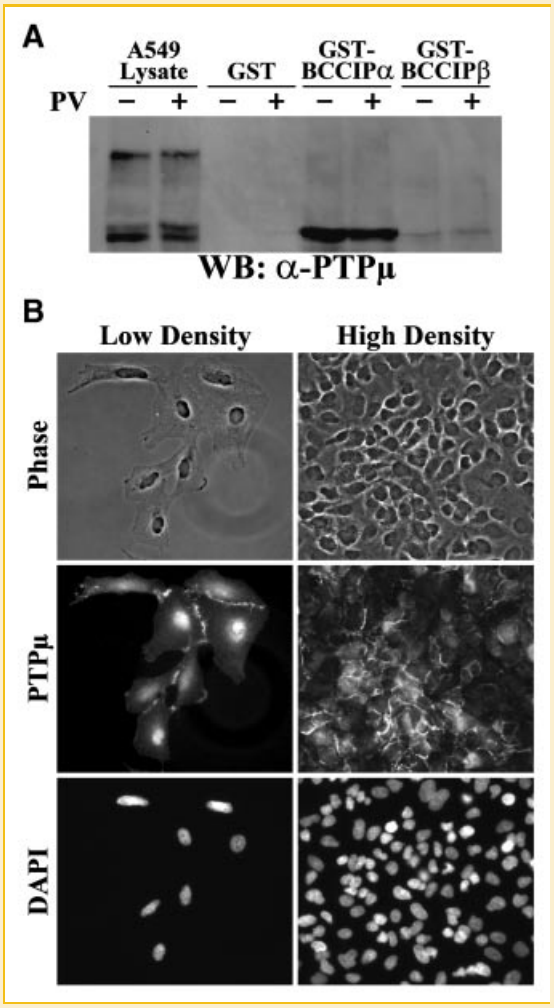


Fig. 5. BCCIP binds endogenous PTPμ. A: A549 cells were treated with control medium (–) or pervanadate (+) for 20 min. Cells were lysed and equal amounts of protein lysate were incubated with GST, GST-BCCIPα and GST-BCCIPβ immobilized on glutathione Sepharose. Associated PTPμ was detected by resolving associated proteins by SDS–PAGE, transferring to nitrocellulose membrane and immunoblotting with an intracellular antibody to PTPμ (SK18). PTPμ is present in the cell as a full length, 200 kDa form and a proteolytically processed form which yields a fragment of ~100 kDa. BCCIPα predominately interacts with the cleaved intracellular form of PTPμ in human cells. B: Immunocytochemistry was performed on A459 cells using an intracellular antibody to PTPμ (SK18). In low density cell cultures PTPμ localizes to nascent cell–cell contacts and a fraction of PTPμ is seen in the nucleus. At high density cell cultures PTPμ is stabilized at cell–cell contacts.

pervanadate prior to making cell lysates. Pervanadate treatment elevates phosphotyrosine levels by inhibiting tyrosine phosphatases. Figure 5A shows that both BCCIPα and BCCIPβ can pull down endogenous PTPμ, however, BCCIPα was much more efficient. The presence of pervanadate had no affect on the interaction. Full length PTPμ, which migrates ~200 kDa on SDS–PAGE, is proteolytically processed by a furin-like protease, generating two fragments of ~100 kDa each, which remain tightly associated by a non-covalent mechanism [Brady-Kalnay and Tonks, 1994]. Therefore, PTPμ immunoreactive bands are recognized at both 200 and 100 kDa on an immunoblot. BCCIP appears to predominately interact with the cleaved, intracellular form of PTPμ (Fig. 5A). These data support the yeast two-hybrid data in that BCCIPα appears to have a greater affinity for PTPμ than BCCIPβ in human cell lines and that BCCIP may preferentially interact with the cleaved, intracellular form of PTPμ.

## INTRACELLULAR PTPμ IS FOUND IN THE NUCLEUS

Recently, the proteolytic cleavage of some RPTPs has been described [Aicher et al., 1997; Anders et al., 2006; Haapasalo et al., 2007]. A common theme among the RPTPs investigated is sequential cleavage resulting in fragments that can localize to distinct areas of the cell, including the nucleus. Proteolytic cleavage of PTPμ has not been studied thoroughly, however, cleavage of a closely related phosphatase, RPTPκ, has been reported [Anders et al., 2006]. Anders et al. show RPTPκ is sequentially cleaved by furin, then ADAM 10 to release an extracellular fragment, followed by a γ-secretase-dependent cleavage within the interior of the lipid bi-layer to release an intracellular fragment that translocates to the nucleus. To test whether related phosphatases were cleaved in a similar manner, Anders et al. used PTPμ in a series of experiments. The results of Anders et al. [2006] suggest PTPμ is proteolytically processed similar to RPTPκ, and that an intracellular PTPμ fragment is generated. This intracellular fragment of PTPμ could potentially translocate to the nucleus but that was not tested by Anders et al. Interestingly, we can see a pool of endogenous PTPμ in the nucleus of A549 lung cancer cells by immunocytochemisty using an antibody directed against the intracellular domain of PTPμ (Fig. 5B). The nuclear staining of PTPμ is most evident in low cell density cultures. Whereas, in high cell-density cultures, PTPμ is localized to cell–cell contacts as previously described [Brady-Kalnay et al., 1995; Gebbink et al., 1995]. To support the notion that the nuclear PTPμ seen by immunocytochemistry is a cleaved intracellular fragment, we made an intracellular fragment of PTPμ (amino acids 765–1451) fused to a GFP tag. When we expressed this construct in A549 cells, it went predominantly to the nucleus (Fig. 6A). We performed immunocytochemistry on A549 cells expressing iPTPμ:765–1451-GFP using an anti-BCCIP antibody to show both PTPμ and BCCIP reside in the nucleus at the same time, demonstrating the potential for PTPμ and BCCIP to interact in the nucleus. Full-length PTPμ fused to GFP localized at cell–cell contacts (Fig. 6B), indicating that addition of the GFP tag did not alter subcellular location. PTPμ could modulate BCCIP activity in the nucleus or perhaps BCCIP could shuttle PTPμ in or out of the nucleus.

## ANTISENSE BCCIP PERTURBS PTPμ FUNCTION

PTPμ function is well characterized in the chick retina where PTPμ has been shown to be a permissive substrate for E8 nasal RGC neurite outgrowth and axon guidance [Burden-Gulley et al., 2002; Ensslen and Brady-Kalnay, 2004; Ensslen-Craig and Brady-Kalnay, 2005]. To determine whether BCCIP regulates PTPμ-mediated neurite outgrowth, we performed Bonhoeffer stripe assays in the presence of BCCIP antisense expression. Stripe assays are used to measure the preference of neurites to grow on different substrates.

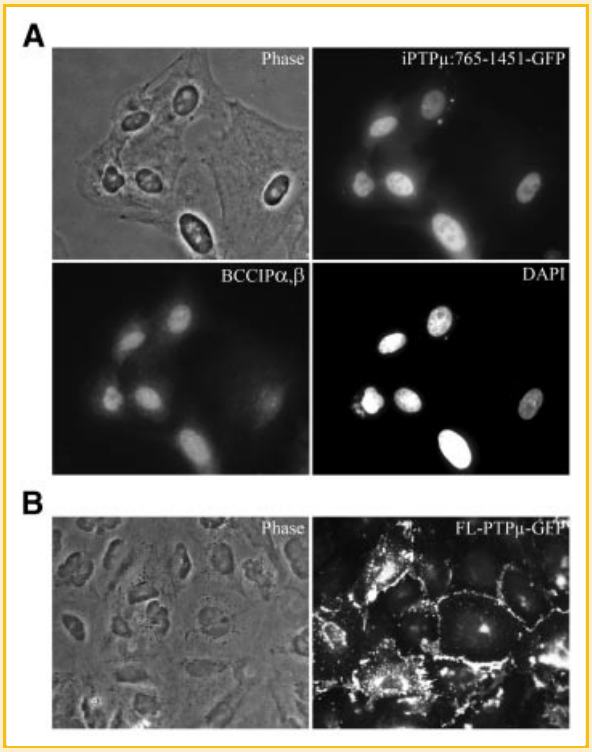


Fig. 6. Exogenously expressed intracellular PTPμ localizes to the nucleus. A: A549 cells were infected with lentivirus encoding an intracellular PTPμ construct, iPTPμ:765–1451-GFP. Immunocytochemistry was performed 24 h post-infection using a primary monoclonal antibody that recognizes both BCCIPα and BCCIPβ and a secondary antibody conjugated to Texas Red. iPTPμ:765–1451-GFP and BCCIP show predominantly nuclear localization, defined by DAPI staining, with some diffuse cytoplasmic staining. B: A549 cells were infected with lentivirus encoding full-length PTPμ-GFP. Images of PTPμ-GFP localization were taken 24 h post-infection and show full-length (FL) PTPμ-GFP is stabilized at cell–cell contacts.

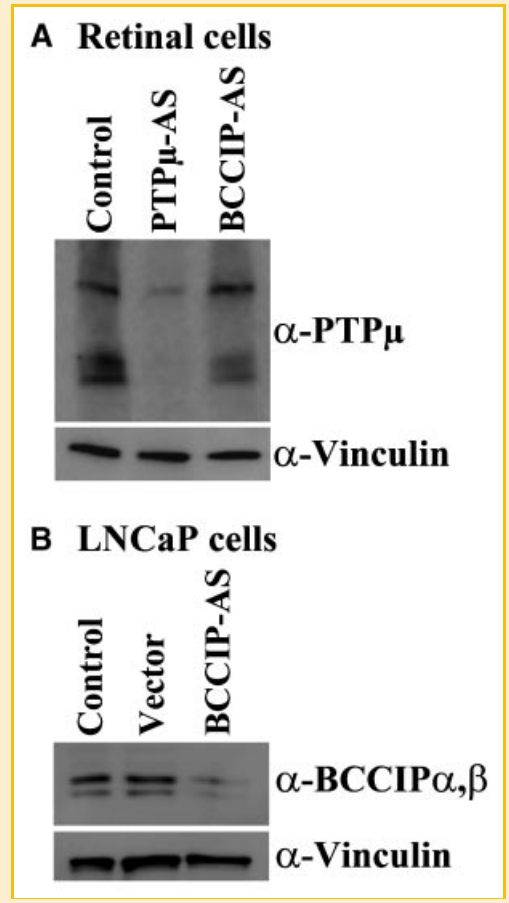


Fig. 7. Antisense downregulation of PTPμ and BCCIP proteins. A: Lysates from E6 retinal cells infected with HSV-antisense PTPμ were separated by SDS–PAGE, transferred to nitrocelllose and immunblotted with an antibody to PTPμ (SK18). Densitometric measurement of the PTPμ bands showed a 60% reduction in PTPμ protein. Antisense BCCIP had little to no effect PTPμ protein levels. B: Lysates from LNCaP cells infected with HSV-antisense BCCIP were separated by SDS–PAGE, transferred to nitrocellulose and immunblotted with an antibody to BCCIP. Densitometric measurement of the BCCIP showed an average 55% reduction of BCCIP protein expression based on three separate experiments. For both A and B, immunoblots were stripped and reprobed with an antibody to vinculin as a protein loading control.

To ensure that the HSV encoding antisense PTPμ and antisense BCCIP reduce PTPμ and BCCIP protein expression, respectively, we measured protein expression of HSV-infected cells by immunoblot. For PTPμ expression, E6 retinal cells were infected with antisense PTPμ-HSV, antisense BCCIP-HSV or uninfected for 24 h. Infection of retinal cells with antisense PTPμ-HSV reduced the overall PTPμ protein levels by 60% (Fig. 7A). This reduction in protein is similar to what we have previously published with the antisense PTPμ-HSV construct [Ensslen et al., 2003; Ensslen-Craig and Brady-Kalnay, 2005; Oblander et al., 2007]. Antisense BCCIP-HSV had little to no effect on PTPμ protein expression. Since there are no available antibodies to BCCIP that recognize chick protein, we determined the efficiency of the antisense BCCIP-HSV, in LNCaP cells because we can detect BCCIP in these human cells with an available antibody and they can be infected with HSV. LNCaP cells were infected with antisense BCCIP-HSV, the HSV vector alone, or uninfected for 24 h. Infection with antisense BCCIP-HSV reduced both BCCIP isoforms by an average of 55% based on three separate experiments (Fig. 7B).

Both PTPμ and laminin are permissive substrates for E8 chick nasal RGCs and neurites grow freely on these substrates (Fig. 8, control). We have previously shown that both downregulation of PTPμ protein via antisense and over-expression of a catalytically inactive PTPμ block neurite outgrowth on a PTPμ substrate [Ensslen-Craig and Brady-Kalnay, 2005]. When antisense BCCIP is added to the cultures, PTPμ is no longer a permissive substrate for the neurites (neurites do not grow on PTPμ lanes), suggesting BCCIP is required for PTPμ's permissive function (Fig. 8). Note that the neurites retain their ability to grow on laminin, demonstrating the specificity of the BCCIP antisense effect for the PTPμ substrate lane. These results suggest BCCIP protein expression is required for PTPμ to promote neurite outgrowth. Green fluoresence protein (GFP) was used as a negative control for viral infection. PTPμ antisense was used as a positive control.

PTPμ is expressed as a gradient in the chick retina with the nasal region having low PTPμ expression and the temporal region having high PTPμ expression [Burden-Gulley et al., 2002]. The differences

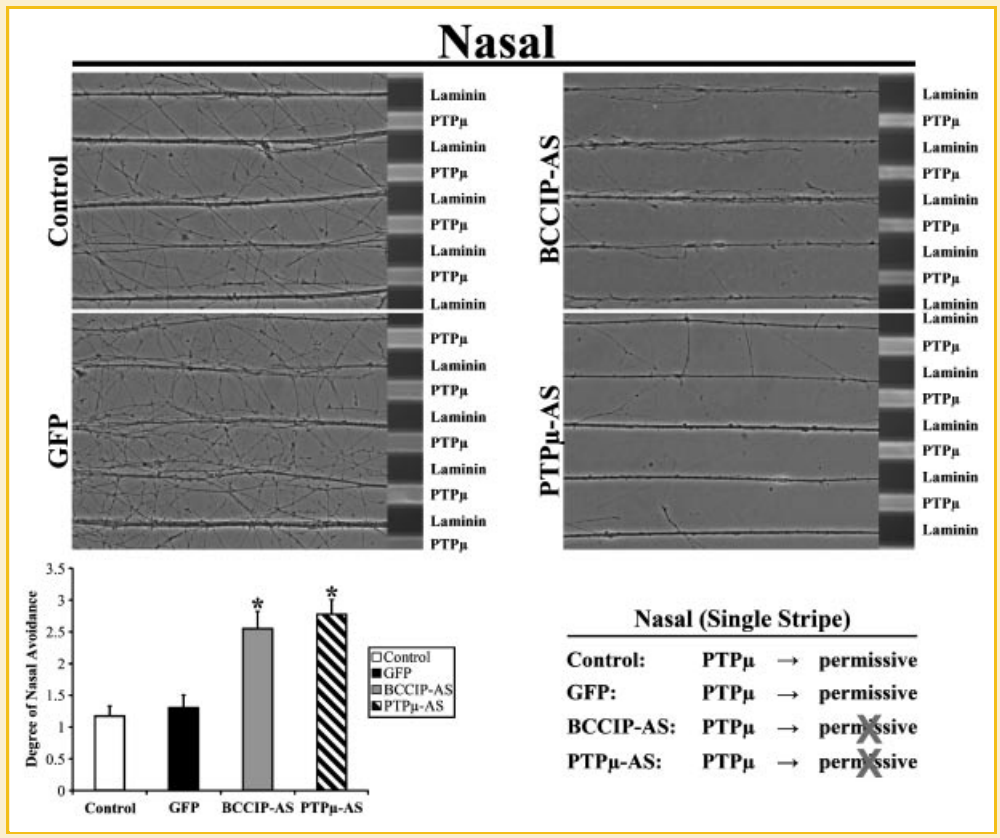


Fig. 8. BCCIP regulates PTPμ-mediated nasal neurite outgrowth. BCCIP antisense expression perturbs PTPμ-mediated neurite outgrowth. Explants from E8 nasal retina were grown on alternating stripes of PTPμ or laminin only lanes. Explants were incubated with GFP-HSV, antisense PTPμ-HSV or antisense BCCIP-HSV virus or without any virus (control). In both the control and GFP-HSV virus control explants, nasal neurites cross freely onto a PTPμ substrate. In the explants incubated with antisense BCCIP, nasal neurites no longer cross onto the PTPμ substrate lanes, or avoid these lanes, suggesting BCCIP is required for a permissive response to the PTPμ substrate in those lanes. Antisense PTPμ-HSV was included as a positive control for nasal neurite avoidance. The average degree of avoidance (mean ± SD) was plotted for each condition as described under Materials and Methods Section. An asterisk denotes a statistically significant change in nasal neurite outgrowth ($P < 0.0001$).

in PTPμ expression yield different outcomes in neurite outgrowth assays. As mentioned above, PTPμ is a permissive substrate for E8 chick nasal RGCs. In contrast, PTPμ is a repulsive substrate for E8 chick temporal RGCs where PTPμ expression is high [Ensslen and Brady-Kalnay, 2004; Ensslen-Craig and Brady-Kalnay, 2005]. To further investigate the role of BCCIP in PTPμ function, we used a mixed stripe assay to determine whether BCCIP was also involved in PTPμ's repulsive activity. Mixed stripe assays combine a permissive substrate (laminin) with a repulsive substrate (PTPμ) [Ensslen-Craig and Brady-Kalnay, 2005]. When the repulsive signal is blocked, the neurites grow on the permissive component (laminin) of that lane. In E8 chick temporal RGCs, PTPμ's repulsive activity is dominant over laminin [Ensslen-Craig and Brady-Kalnay, 2005], therefore, under control conditions, E8 chick temporal RGCs only grow on laminin lanes and avoid lanes that contain both laminin and PTPμ (mixed stripes, Fig. 9). This confirms PTPμ is repulsive to temporal RGCs and that PTPμ signaling is dominant over laminin. As expected, the presence of PTPμ antisense blocked the repulsive activity of PTPμ in the mixed stripes, allowing the neurites to grow on the permissive laminin substrate also present in the lane as previously demonstrated [Ensslen-Craig and Brady-Kalnay, 2005]. When cultures are grown in the presence of BCCIP antisense, the lanes containing both PTPμ and laminin are no longer repulsive (Fig. 9, BCCIP-AS), indicating PTPμ function and signaling has been impaired. GFP and PTPμ antisense were used as negative and positive controls, respectively.

## DISCUSSION

While many investigators have studied RPTP functions, it has been more problematic to identify signals that are directly transduced by RPTPs in response to cell adhesion or "ligand" binding. The identification of RPTP signaling pathways represents a major area of investigation in this field. To identify additional PTPμ interacting proteins, we used a modified yeast two-hybrid screen [Keegan and Cooper, 1996; Lioubin et al., 1996] to isolate proteins that bind PTPμ in the presence of the Src tyrosine kinase. In this system, proteins expressed in yeast are phosphorylated by an exogenously expressed constitutively active Src tyrosine kinase. We isolated BCCIPα in this screen as a protein that binds to the first phosphatase domain of PTPμ. This interaction was confirmed in vitro. Furthermore, we have

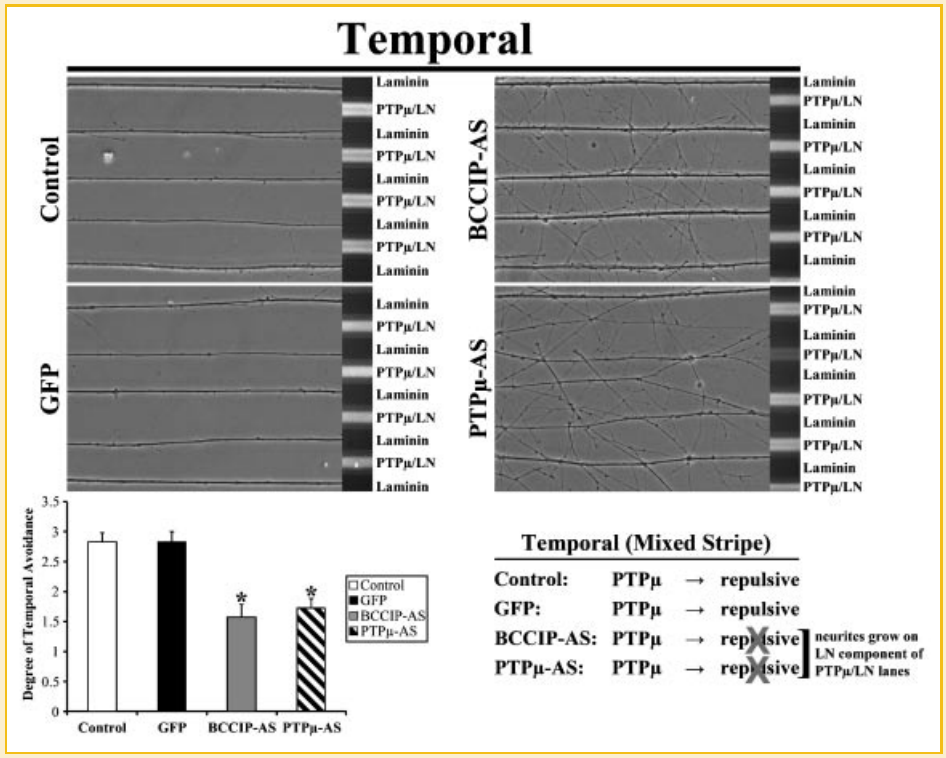


Fig. 9. BCCIP is required for PTPμ-dependent temporal repulsion. Explants from E8 temporal retina were grown on alternating stripes of PTPμ mixed with laminin (PTPμ/LN) versus laminin only lanes. Explants were incubated with or without virus as described above and neurites were evaluated for crossing from the laminin lanes to the PTPμ mixed substrate lanes. In both the control and GFP-HSV virus control explants, neurites avoid the PTPμ mixed substrate lanes. In the explants incubated with antisense BCCIP, neurites crossed the PTPμ-containing lanes, suggesting BCCIP is involved in the regulation of PTPμ-dependent repulsion of temporal neurites. Antisense PTPμ-HSV was included as a positive control for blocking temporal repulsion. The average degree of avoidance (mean ± SD) was plotted for each condition as described under Materials and Methods Section. An asterisk denotes a statistically significant change in temporal neurite repulsion ($P < 0.0001$).

demonstrated that BCCIPα is a substrate for both the Src tyrosine kinase and the PTPμ tyrosine phosphatase in vitro.

BCCIP is a relatively novel protein. It was identified independently by two groups as a p21 [Ono et al., 2000] and BRCA2 [Liu et al., 2001] interacting protein. BCCIP has been shown to inhibit cell growth [Liu et al., 2001; Meng et al., 2004a] and to regulate p21 expression in a p53-dependent manner [Meng et al., 2004b]. In addition, BCCIP plays a role in homologous DNA repair [Lu et al., 2005, 2007], chromosome stability [Meng et al., 2007a], and p53 transactivation [Meng et al., 2007b]. A BCCIP ortholog has been identified in *Ustilago maydis* and is implicated in DNA repair and homologous recombination like its mammalian counterpart [Mao et al., 2007]. A yeast homolog of BCCIP was cloned and named Bcp1 [Audhya and Emr, 2003]. Bcp1 interacts with PI4,5$P_2$ phosphoinositide kinase (PI4P 5-kinase) in yeast. The PI4P 5-kinase shuttles from the plasma membrane to the nucleus, and this process is controlled by phosphorylation [Audhya and Emr, 2003]. Bcp1 binds PI4 5-kinase and controls its nuclear export [Audhya and Emr, 2003]. These authors hypothesize that the physiological function of Bcp1 is to control nuclear export of signaling proteins that regulate cell growth, including PI4P 5-kinase, p21/Cdk2 and BRCA-2. Our data suggest that there are sufficient cytoplasmic pools of BCCIPα and BCCIPβ isoforms. We hypothesize that BCCIP may shuttle between the cytosolic and nuclear compartment in response to signal transduction. Based on recent data demonstrating some RPTPs including PTPμ undergo multiple cleavage events generating fragments that may enter the nucleus [Anders et al., 2006; Haapasalo et al., 2007] it is exciting to postulate that BCCIP and an intracellular cleavage product of PTPμ may translocate to the nucleus in response to a cell signaling event.

Loss of contact inhibition of growth and adhesion-based signals are necessary for cancer progression. It is interesting to note that both BCCIP and PTPμ expression levels change in cancer cells [Liu et al., 2001; Hellberg et al., 2002]. BCCIP expression is altered in some breast and brain tumor cells [Liu et al., 2001], and BCCIP is down regulated in both cancers of the kidney [Meng et al., 2003] and astrocyctic brain tumors [Meng et al., 2007a]. Normal prostate cells express PTPμ whereas LNCaP prostate carcinoma cells do not [Hellberg et al., 2002]. We used this cancer cell model system to analyze the role of PTPμ in the regulation of cell adhesion. Normal epithelial architecture is maintained by E-cadherin, a homophilic cell–cell adhesion molecule. Disrupting E-cadherin or its associated proteins, catenins, correlates with invasion and metastasis [Christofori and Semb, 1999; Van Aken et al., 2001]. Changes in E-cadherin, N-cadherin, α-catenin, β-catenin, plakoglobin and p120 catenin expression have been observed in many types of carcinomas [Van Aken et al., 2001; Kallakury et al., 2001a,b]. PTPμ has previously been shown to interact with the E-cadherin complex

[Brady-Kalnay et al., 1995, 1998]. LNCaP cells express normal levels of E-cadherin and catenins, but do not mediate either PTPμ- or E-cadherin-dependent adhesion [Hellberg et al., 2002]. Re-expression of PTPμ restored cell adhesion to both PTPμ and to E-cadherin [Hellberg et al., 2002] demonstrating a role for PTPμ in the regulation of E-cadherin function. LNCaP cells were used in this manuscript and express both BCCIPα and β. In future studies, we will investigate how PTPμ-dependent cell adhesion regulates signaling and perhaps contact inhibition of growth via BCCIP.

Recently, BCCIP has been shown to promote p53 transcription activity [Meng et al., 2007b]. Down regulation of BCCIP protein abrogates p53-mediated upregulation of p21 in response to radiation. The mechanism by which BCCIP appears to regulate p53 activity is by facilitating the formation of active p53 tetramers [Meng et al., 2007b]. This is of interest to us because p53 can regulate neurite outgrowth [Bacsi et al., 2005; Di Giovanni et al., 2006]. PTPμ is well established as both a permissive and repulsive cue for RGC neurite outgrowth [Burden-Gulley et al., 2002; Ensslen-Craig and Brady-Kalnay, 2005]. In addition, PTPμ is required for both N-cadherin [Burden-Gulley and Brady-Kalnay, 1999] and E-cadherin [Oblander et al., 2007]-mediated neurite outgrowth. The data presented in this manuscript clearly demonstrate BCCIP is required for PTPμ-mediated neurite outgrowth. Future studies will investigate the molecular mechanism by which BCCIP regulates PTPμ-dependent neurite outgrowth and cell-cell adhesion.

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## REFERENCES

Aicher B, Lerch MM, Müller T, Schilling J, Ulrich A. 1997. Cellular redistribution of protein tyrosine phosphatases LAR and PTPs by inducible proteolytic processing. J Cell Biol 138:681–696.

Anders L, Mertins P, Lammich S, Murgia M, Hartmann D, Saftig P, Haass C, Ullrich A. 2006. Furin-, ADAM 10-, and γ-secretase-mediated cleavage of a receptor tyrosine phosphatase and regulation of β-catenin's transcriptional activity. Mol Cell Biol 26:3917–3934.

Audhya A, Emr SD. 2003. Regulation of PI4,5P2 synthesis by nuclear-cytoplasmic shuttling of the Mss4 lipid kinase. EMBO J 22:4223–4236.

Bacsi A, Stanton GJ, Hughes TK, Kruze M, Boldogh I. 2005. Colostrinin-driven neurite outgrowth requires p53 activation in PC12 cells. Cell Mol Neurobiol 27:1123–1139.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Brady-Kalnay SM, Tonks NK. 1993. Purification and characterization of the human protein tyrosine phosphatase, PTP mu, from a baculovirus expression system. Mol Cell Biochem 127-128:131–141.

Brady-Kalnay SM, Tonks NK. 1994. Identification of the homophilic binding site of the receptor protein tyrosine phosphatase PTP mu. J Biol Chem 269:28472–28477.

Brady-Kalnay SM, Flint AJ, Tonks NK. 1993. Homophilic binding of PTP mu, a receptor-type protein tyrosine phosphatase, can mediate cell-cell aggregation. J Cell Biol 122:961–972.

Brady-Kalnay SM, Rimm DL, Tonks NK. 1995. Receptor protein tyrosine phosphatase PTPmu associates with cadherins and catenins in vivo. J Cell Biol 130:977–986.

Brady-Kalnay SM, Mourton T, Nixon JP, Pietz GE, Kinch M, Chen H, Brackenbury R, Rimm DL, Del Vecchio RL, Tonks NK. 1998. Dynamic interaction of PTPmu with multiple cadherins in vivo. J Cell Biol 141: 287–296.

Burden-Gulley SM, Brady-Kalnay SM. 1999. PTPmu regulates N-cadherin-dependent neurite outgrowth. J Cell Biol 144:1323–1336.

Burden-Gulley SM, Ensslen SE, Brady-Kalnay SM. 2002. Protein tyrosine phosphatase-mu differentially regulates neurite outgrowth of nasal and temporal neurons in the retina. J Neurosci 22:3615–3627.

Christofori G, Semb H. 1999. The role of the cell-adhesion molecule E-cadherin as a tumour- suppressor gene. Trends Biochem Sci 24:73–76.

Di Giovanni S, Knights CD, Roa M, Yakovlev A, Beers J, Catania J, Avantaggiati ML, Faden AI. 2006. The tumor suppressor protein p53 is required for neurite outgrowth and axon regeneration. EMBO J 25:4084–4096.

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. 1998. A third-generation lentivirus vector with a conditional packaging system. J Virol 72:8463–8471.

Ensslen SE, Brady-Kalnay SM. 2004. PTPmu signaling via PKCdelta is instructive for retinal ganglion cell guidance. Mol Cell Neurosci 25:558–571.

Ensslen SE, Rosdahl JA, Brady-Kalnay SM. 2003. The receptor protein tyrosine phosphatase mu, PTPmu, regulates histogenesis of the chick retina. Dev Biol 264:106–118.

Ensslen-Craig SE, Brady-Kalnay SM. 2005. PTPmu expression and catalytic activity are required for PTP mu-mediated neurite outgrowth and repulsion. Mol Cell Neurosci 28:177–188.

Gebbink MF, Verheijen MH, Zondag GC, van Etten I, Moolenaar WH. 1993a. Purification and characterization of the cytoplasmic domain of human receptor-like protein tyrosine phosphatase RPTP mu. Biochemistry 32: 13516–13522.

Gebbink MF, Zondag GC, Wubbolts RW, Beijersbergen RL, van Etten I, Moolenaar WH. 1993b. Cell-cell adhesion mediated by a receptor-like protein tyrosine phosphatase. J Biol Chem 268:16101–16104.

Gebbink MF, Zondag GC, Koningstein GM, Feiken E, Wubbolts RW, Moolenaar WH. 1995. Cell surface expression of receptor protein tyrosine phosphatase RPTP mu is regulated by cell-cell contact. J Cell Biol 131: 251–260.

Golemis EA. 1997. Interaction trap/two-hybrid system to identify interacting proteins. In: Brent R, Current protocols in molecular biology. New York: John Wiley & Sons, Inc. pp 20.1.1–20.1.35.

Gumbiner BM. 2005. Regulation of cadherin-mediated adhesion in morphogenesis. Nat Rev Mol Cell Biol 6:622–634.

Haapasalo A, Kim DY, Carey BW, Turunen MK, Pettingell WH, Kovacs DM. 2007. Presenilin/gamma-secretase-mediated cleavage regulates association of leukocyte-common antigen-related (LAR) receptor tyrosine phosphatase with beta-catenin. J Biol Chem 282:9063–9072.

Hellberg CB, Burden-Gulley SM, Pietz GE, Brady-Kalnay SM. 2002. Expression of the receptor protein-tyrosine phosphatase, PTPmu, restores E-cadherin-dependent adhesion in human prostate carcinoma cells. J Biol Chem 277:11165–11173.

Hiscox S, Jiang WG. 1998. Association of PTPmu with catenins in cancer cells: A possible role for E-cadherin. Int J Oncol 13:1077–1080.

Kallakury BV, Sheehan CE, Ross JS. 2001a. Co-downregulation of cell adhesion proteins alpha- and beta-catenins, p120CTN, E-cadherin, and CD44 in prostatic adenocarcinomas. Hum Pathol 32:849–855.

Kallakury BV, Sheehan CE, Winn-Deen E, Oliver J, Fisher HA, Kaufman RP, Jr., Ross JS. 2001b. Decreased expression of catenins (alpha and beta), p120 CTN, and E- cadherin cell adhesion proteins and E-cadherin gene promoter methylation in prostatic adenocarcinomas. Cancer 92:2786–2795.

Keegan K, Cooper JA. 1996. Use of the two hybrid system to detect the association of the protein-tyrosine-phosphatase, SHPTP2, with another SH2-containing protein, GRB7. Oncogene 12:1537–1544.

Kuroda S, Fukata M, Nakagawa M, Fujii K, Nakamura T, Ookubo T, Izawa I, Nagase T, Nomura N, Tani H, Shoji I, Matsuura Y, Yonehara S, Kaibuchi K. 1998. Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin- mediated cell-cell adhesion. Science 281:832–835.

Lagenaur C, Lemmon V. 1987. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc Natl Acad Sci USA 84:7753–7757.

Li Z, Kim SH, Higgins JM, Brenner MB, Sacks DB. 1999. IQGAP1 and calmodulin modulate E-cadherin function. J Biol Chem 274:37885–37892.

Lilien J, Balsamlo J. 2005. The regulation of cadherin-mediated adhesion by tyrosine phosphorylation. Curr Opin Cell Biol 17:459–465.

Lioubin MN, Algate PA, Schickwann T, Carlberg K, Aebersold R, Rohrschneider LR. 1996. p150ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. Genes Dev 10:1084–1095.

Liu J, Yuan Y, Huan J, Shen Z. 2001. Inhibition of breast and brain cancer cell growth by BCCIPalpha, an evolutionarily conserved nuclear protein that interacts with BRCA2. Oncogene 20:336–345.

Lu H, Guo X, Meng X, Liu J, Allen C, Wray J, Nickoloff JA, Shen Z. 2005. The BRCA2-interacting protein BCCIP functions in RAD51 and BRCA2 focus formation and homologous recombinational repair. Mol Cell Biol 25:1949–1957.

Lu H, Yue J, Meng X, Nickoloff JA, Shen Z. 2007. BCCIP regulates homologous recombination by distinct domians and supresses spontaneous DNA damage. Nucleic Acids Res 35:7160–7170.

Machida K, Mayer BJ, Nollau P. 2003. Profiling the global tyrosine phosphorylation state. Mol Cell Proteomics 2:215–233.

Mao N, Zhou Q, Kojic M, Perez-Martin J, Holloman WK. 2007. Ortholog of BRCA2-interacting protein BCCIP controls morphogenetic responses during DNA replication stress in Ustilago maydis. DNA Repair (Amst) 6:1651–1660.

Meng X, Liu J, Shen Z. 2003. Genomic structure of the human BCCIP gene and its expression in cancer. Gene 302:139–146.

Meng X, Liu J, Shen Z. 2004a. Inhibition of G1 to S cell cycle progression by BCCIP beta. Cell Cycle 3:343–348.

Meng X, Lu H, Shen Z. 2004b. BCCIP functions through p53 to regulate the expression of p21Waf1/Cip1. Cell Cycle 3:1457–1462.

Meng X, Fan J, Shen Z. 2007a. Roles of BCCIP in chromosome stability and cytokinesis. Oncogene 26:6253–6260.

Meng X, Yue J, Zhihe L, Shen Z. 2007b. Abrogation of the transactivation activity of p53 by BCCIP down-regulation. J Biol Chem 282:1570–1576.

Mourton T, Hellberg CB, Burden-Gulley SM, Hinman J, Rhee A, Brady-Kalnay SM. 2001. The PTPmu protein-tyrosine phosphatase binds and recruits the scaffolding protein RACK1 to cell-cell contacts. J Biol Chem 276:14896–14901.

Oblander SA, Ensslen-Craig SE, Longo FM, Brady-Kalnay SM. 2007. E-cadherin promotes retinal ganglion cell neurite outgrowth in a protein tyrosine phosphatase-mu dependent manner. Mol Cell Neurosci 34:481–492.

Ono T, Kitaura H, Ugai H, Murata T, Yokoyama KK, Iguchi-Ariga SM, Ariga H. 2000. TOK-1, a novel p21Cip1-binding protein that cooperatively enhances p21-dependent inhibitory activity toward CDK2 kinase. J Biol Chem 275: 31145–31154.

Pawson T. 2002. Regulation and targets of receptor tyrosine kinases. Eur J Cancer 38 (Suppl 5): S3–S10.

Phillips-Mason PJ, Gates TJ, Major DL, Sacks DB, Brady-Kalnay SM. 2006. The receptor protein-tyrosine phosphatase PTPμ interacts with IQGAP1. J Biol Chem 281:4903–4910.

Rosdahl JA, Mourton TL, Brady-Kalnay SM. 2002. Protein kinase C delta (PKCdelta) is required for protein tyrosine phosphatase mu (PTPmu)-dependent neurite outgrowth. Mol Cell Neurosci 19:292–306.

Rosdahl JA, Ensslen SE, Niedenthal JA, Brady-Kalnay SM. 2003. PTPμ-dependent growth cone rearrangement is regulated by Cdc42. J Neurobiol 56:199–208.

Sui XF, Kiser TD, Hyun SW, Angelini DJ, Del Vecchio RL, Young BA, Hasday JD, Romer LH, Passaniti A, Tonks NK, Goldblum SE. 2005. Receptor protein tyrosine phosphatase μ regulates the paracellular pathway in human lung microvascular endothelia. Am J Pathol 166:1247–1258.

Tonks NK. 2006. Protein tyrosine phosphatases: From genes, to function, to disease. Nat Rev Mol Cell Biol 7:833–846.

Van Aken E, De Wever O, Correia da Rocha AS, Mareel M. 2001. Defective E-cadherin/catenin complexes in human cancer. Virchows Arch 439:725–751.

Vielmetter J, Stolze B, Bonhoeffer F, Stuermer CA. 1990. In vitro assay to test differential substrate affinities of growing axons and migratory cells. Exp Brain Res 81:283–287.

Walter J, Henke-Fahle S, Bonhoeffer F. 1987. Avoidance of posterior tectal mambranes by temporal retinal axons. Development 101:909–913.

Zondag GC, Reynolds AB, Moolenaar WH. 2000. Receptor protein-tyrosine phosphatase RPTPmu binds to and dephosphorylates the catenin p120(ctn). J Biol Chem 275:11264–11269.