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Differential Induction of Glioblastoma Migration and Growth by Two Forms of Pleiotrophin*

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Glioblastoma is the most common malignant brain tumor of adults and one of the most lethal cancers. The secreted growth factor pleiotrophin (PTN) promotes glioblastoma migration and proliferation, initiating its oncogenic activities through two cell surface receptors, the protein tyrosine phosphatase receptor ζ (PTPRZ1) and the anaplastic lymphoma kinase (ALK), respectively. Here, we report on the presence and purification of two naturally occurring forms of PTN (18 and 15 kDa) that differentially promote glioblastoma migration and proliferation. Using a panel of glioblastoma cell lines, including low passage patient-derived cultures, we demonstrate that PTN15 promotes glioblastoma proliferation in an ALK-dependent fashion, whereas immobilized PTN18 promotes haptotactic migration of glioblastoma cells in a PTPRZ1-dependent fashion. Mass spectrometric analysis indicated that PTN15 differs from PTN18 by processing of 12 C-terminal amino acids. To demonstrate clinical relevance, we show that PTN15, PTN18, and PTPRZ1 are significantly overexpressed in glioblastoma relative to normal brain at both mRNA and protein levels using microarray, Western blot, and tissue microarray analyses on human tumors. These results indicate that the PTN18-PTPRZ1 and the PTN15-ALK signaling pathways represent potentially important therapeutic targets for glioblastoma invasion and growth.

Glioblastoma is the most frequent and lethal primary brain tumor of adults, with a median survival time of 12 months from the time of diagnosis despite aggressive surgery, radiation, and chemotherapy. Although refractory to standard treatments, glioblastomas are well suited for targeted molecular therapies (1). The characterization of molecules or pathways critical to glioblastoma pathogenesis may thus lead to the discovery of novel targets for therapy.

The secreted growth factor PTN[1] (also called HARP, HB-GAM, HBNF, and OSF-1) may represent such a potential target.

PTN is a heparin-binding, developmentally regulated protein that is expressed in the embryonic and postnatal nervous system (2–5) and has been shown to be overexpressed in a number of cancers, including glioblastomas (6–11). A variety of biological activities have been attributed to PTN including mitogenesis, angiogenesis, neurite outgrowth, and cell migration (12). PTN promotes glioblastoma growth via the receptor tyrosine kinase ALK in a process that can be mediated via the phosphatidylinositol 3-kinase and MAPK signaling pathways (13, 14). PTN also engages a second receptor, the receptor tyrosine phosphatase PTPRZ1 (also termed RPTPβ and PTPζ) (15, 16), and functional data suggest that this interaction may promote glioblastoma migration (17). Like PTN, both ALK and PTPRZ1 are predominantly expressed in the developing central nervous system (18–21) and have also been shown to be overexpressed in glioblastomas (11, 13, 17).

To date, the molecular mechanisms underlying PTN-mediated invasion and proliferation through these receptors have yet to be fully elucidated. Because the two receptor pathways have not been closely studied together, it remains unclear whether their signaling cascades converge or act independently to promote glioblastoma progression, especially because they share the same ligand yet have seemingly antagonistic enzymatic functions. Furthermore, recent work suggests that differences in cellular processing or post-translational modification of PTN may be responsible for some of its different functional effects (12). We now describe the isolation and characterization of two naturally occurring forms of PTN with migratory masses of 18 and 15 kDa. We show that PTN15 specifically promotes glioblastoma proliferation in an ALK-dependent fashion, whereas PTN18 promotes glioblastoma migration in a PTPRZ1-dependent fashion, and that the two forms differ by post-translational processing of 12 C-terminal amino acids. Finally, we demonstrate that PTN15, PTN18, and

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[1] The abbreviations used are: PTN, pleiotrophin; ePTN, engineered pleiotrophin; PTPRZ1, protein tyrosine phosphatase receptor ζ; ALK, anaplastic lymphoma kinase; MAPK, mitogen-activated protein kinase; RT, reverse transcription; HPLC, high pressure liquid chromatography; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; BSA, bovine serum albumin; HEK, human embryonic kidney; TBST, Tris-buffered saline/Tween 20.

PTPRZ1 are highly and consistently overexpressed in glioblastoma patient tumors, suggesting critical roles for PTN15-ALK and PTN18-PTPRZ1 signaling in promoting glioblastoma proliferation and invasion.

EXPERIMENTAL PROCEDURES

Cell Lines and Tissue Culture—The human glioblastoma cell lines LN229, T98G, U87MG, and U138MG were purchased from the American Type Culture Collection (Manassas, VA). U373MG was provided by the European Collection of Cell Cultures through Sigma. Low passage (<15) primary human glioblastoma lines were derived from resected patient tumors as described previously (22). HEK 293T cells were a kind gift from Dr. M. Teitell (UCLA). All cells were routinely maintained in minimum essential medium containing 10% fetal bovine serum (Omega Scientific, Tarzana, CA), 1× penicillin-streptomycin-glutamine solution (Invitrogen), 1× non-essential amino acids, 1 mM sodium pyruvate, and 0.15% sodium bicarbonate (Mediatech, Herndon, VA) and grown at 37 °C in the presence of 5% CO_2. For cell stimulation experiments, seeded cells were allowed to adhere overnight before serum starvation for 48 h. Cells were then treated with purified PTN (described below) or commercial PTN (R&D Systems, Minneapolis, MN) at the indicated amounts and times or challenged with serum-containing medium for 10 min.

RT-PCR—RT-PCR was performed as described previously (23). Briefly, first-strand cDNA was generated by reverse transcribing 2 μg of total RNA that was isolated using TRIzol reagent (Invitrogen). 2 μl of cDNA was then used as template in a 50 μl PCR containing Platinum *Taq* Hi Fidelity polymerase and the PCRx Enhancer System (Invitrogen) according to the manufacturer's suggestions. Primers (IDT DNA, Coralville, IA) spanning the entire open reading frame were as follows: for PTN, 5′-GAGCGCCAGAGAGGACGTTT-3′ and 5′-TCCTGTTTGCTGATGTCCTTTT-3′; for PTPRZ1, 5′-ACCGTCTGGAAATGCGAA-3′ and 5′-AACTAGACTGATTTTCCTGCC-3′; and for ALK, 5′-GGCCCGGAGAGCAGTGTAAA-3′ and 5′-CCAGGCTGGTTCATGCTATT-3′. 5 μl of each reaction was then run on 2% (PTN) or 1% (PTPRZ1 and ALK) agarose gels and stained with ethidium bromide.

Western Blotting and Immunoprecipitation—Cells were harvested in ice-cold lysis buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 10 mM EDTA, 1.5 mM $MgCl_2$, 1% Triton X-100, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 20 mM sodium pyrophosphate, and 20 mM sodium fluoride), homogenized through an 18-gauge needle five times each, and then cleared by centrifugation at 14,000 rpm for 15 min at 4 °C. Protein concentrations were determined with the BCA protein assay (Pierce), and usually 20 μg of each protein sample was loaded onto 10% polyacrylamide gels for SDS-PAGE. Following transfer to nitrocellulose membranes (Amersham Biosciences), blots were blocked in 5% milk in TBST for 1 h at room temperature and then incubated with primary antibodies at 4 °C overnight. Monoclonal antibodies against phosphorylated Akt (Ser^{473}) and polyclonal antibodies against phosphorylated p44/42 MAPK (Thr^{202}/Tyr^{204}) (Cell Signaling Technology, Beverly, MA), as well as rabbit polyclonal ALK antibodies (Zymed Laboratories Inc.), were used at 1:000 dilution. Monoclonal anti-PTPRZ1 (RPTPβ) antibodies (BD Transduction Laboratories) were used at 1:500 dilution, whereas monoclonal anti-β-tubulin (Sigma) and anti-V5 (Invitrogen) antibodies were used at 1:5000 dilution. After washing blots in TBST, the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) diluted 1:2000 were added for 1 h at room temperature, and bands were detected with ECL (Amersham Biosciences). For analysis of PTN expression, 15 μl of each media sample conditioned for 48 h was subjected to SDS-PAGE on 15% polyacrylamide gels, the proteins were transferred as described above, and the blots were blocked in 2% horse serum in TBST. Goat anti-PTN antibodies (R&D Systems, Minneapolis, MN) were diluted 1:500 in 2% horse serum and incubated overnight. For immunoprecipitation, 0.5–1.5 mg of protein lysate was incubated overnight with gentle rocking at 4 °C with a mixture of goat polyclonal (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal (Zymed Laboratories Inc.) anti-ALK antibodies diluted at 1:150 each. A 50:50 mixture of protein A-agarose and protein G-agarose beads (Pierce) was then added and incubated for 3 h at 4 °C. The beads were washed in lysis buffer five times and resuspended in SDS-PAGE sample buffer, and the eluted proteins were boiled and subjected to electrophoresis and Western blotting with anti-phospho-tyrosine antibodies (P-Tyr-100; Cell Signaling Technology).

PTN Clones and Transfection—Full-length wild type PTN cDNA was generated by RT-PCR from U87MG total RNA as described above and then cloned into the EcoRI site of the pcDNA3.1 expression vector (Invitrogen). The resulting construct was designated pcDNA3.1-PTN18. Other PTN construct variants were generated by directional cloning of PCR products into pcDNA3.1D/V5-His-TOPO (Invitrogen) according to the manufacturer's instructions. Briefly, pcDNA3.1-PTN18-V5 was created by PCR-amplifying a full-length PTN cDNA lacking the endogenous stop codon and fused in-frame with the 3′ V5-His epitope tag. pcDNA3.1-PTN15 was engineered using a PCR primer to insert a stop codon 36 bases upstream of the endogenous stop codon, translating into a 3′ truncation of 12 amino acids. pcDNA3.1-PTN15-V5 was generated in the same manner, except the artificial stop codon was omitted, and the truncated cDNA was directly cloned in-frame with the 3′ V5-His tag. Orientation and sequences of all constructs were verified by DNA sequencing. HEK 293T cells were transfected with the various PTN constructs using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer's protocol.

Purification of PTN from Conditioned Media—Approximately 2 liters of conditioned media was collected from HEK 293T cells 72 h after being transfected with pcDNA3.1-PTN18 or pcDNA3.1-PTN15 and purified according to Ref. 24, with modifications. After adjusting the media to 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl and clearing the media through a 0.2 μm filter, the media were loaded onto a 5-ml HiTrap heparin HP column and washed with 80 ml of 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and the bound proteins were eluted with 20 ml of 50 mM Tris-HCl, pH 7.5, 1 M NaCl. The eluate was then equilibrated to 50 mM Tris-HCl, pH 7.5, 0.25 M NaCl; concentrated down to 400 μl; and then further fractionated by injecting 200-μl volumes into a TosoHaas (Montgomeryville, PA) TSK-gel SP-5PW sulfopropyl HPLC column (7.5 mm × 7.5 cm) on an HP-1090 HPLC system (Hewlett Packard, Palo Alto, CA) equipped with a ChemStation and a diode array detector. Separation was achieved using a linear gradient from 0.45 M NaCl to 2 M NaCl in 50 mM Tris-HCl, pH 7.5, for 30 min with a flow rate of 0.5 ml/min. Fractions under chromatographic peaks were collected and stored at −20 °C until further analysis.

Cell Proliferation Assay—Cell growth was measured using the WST-1 Cell Proliferation Assay Kit (Chemicon, Temecula, CA) according to the manufacturer's protocol. Briefly, 2500–5000 cells (depending on cell line) per well were seeded in a 96-well tissue culture plate, allowed to adhere overnight, and then serum-starved for 48 h before treatment with the indicated amounts of PTN. Cells were then incubated for an additional 48 h before measuring growth.

Haptotactic and Chemotactic Assays—For haptotactic assays, 6-well Transwell polycarbonate membrane inserts with 8.0-μm pores (CoStar, Cambridge, MA) were coated from the bottom with 5 μg/ml PTN or 50 μg/ml BSA control diluted in phosphate-buffered saline. After adding serum-free minimum essential medium to the lower compartments, 5×10^5 cells in serum-free minimum essential medium/BSA (5 mg/ml) were seeded onto the upper chambers and incubated for 6 h at 37 °C in the presence of 5% CO_2. Non-migrated cells on the upper surface were then removed with cotton swabs, and the remaining migrated cells on the lower surface were fixed in 4% paraformaldehyde and stained with 0.25% crystal violet. Migrated cells were quantitated by imaging 5 random fields/filter on an Olympus BX61 microscope at ×40 magnification and then counting the number of cells from each photomicrograph using the particle separator and colorimetric detection features of the accompanying Olympus MicroSuite software. For chemotactic assays, the filters were left uncoated, but instead, PTN was added into the serum-free media of the lower compartment. Cells were then seeded and incubated, and filters were fixed, stained, and counted as described for the haptotactic assays.

N-terminal Sequence and Mass Spectrometric Analyses—For protein sequence analysis, an aliquot of each sample was run on 15% SDS-PAGE, and protein bands were transferred onto a polyvinylidene difluoride membrane according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). After staining and destaining with Coomassie Blue, protein bands were cut out and directly loaded for N-terminal sequence analysis on a Procise 494 automatic sequencer (Applied Biosystems). MALDI-MS and electrospray LC-MS analyses were performed as described previously (25, 26). For MALDI-MS, an aliquot of each protein sample was mixed with matrix (sinapinic acid; Sigma) for analysis on a Voyager DE-PRO work station (Applied Biosystems). For LC-MS, purified PTN fractions were injected into a reverse phase HPLC column on an HP-1090 system directly connected on-line to an LCQ electrospray ion-trap mass spectrometer (Finnigan, San Jose, CA).

Oligonucleotide Microarrays—Gene expression profiling with Affymetrix high density oligonucleotide microarrays was performed and analyzed as described previously (27, 28). 10 μg of total RNA was

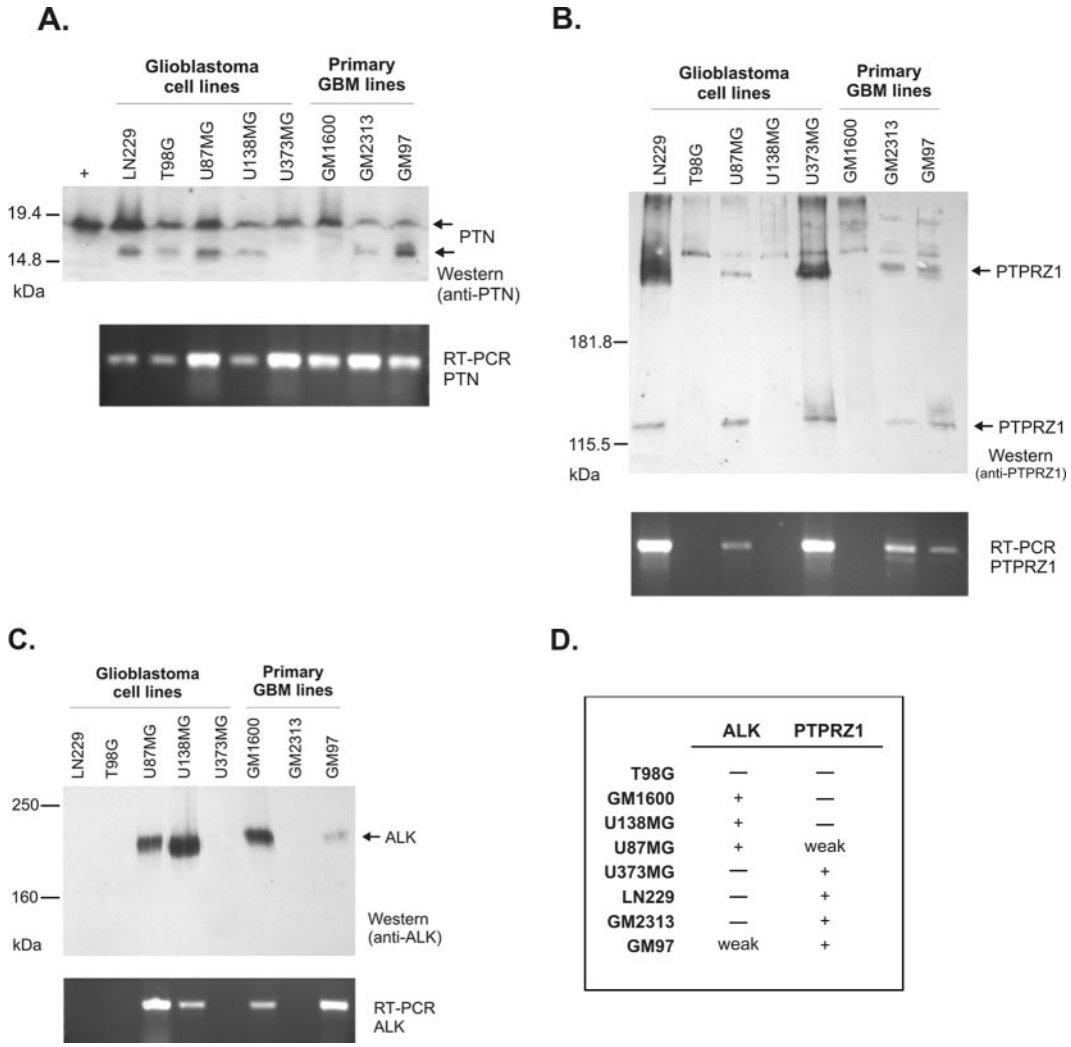

FIG. 1. **PTN, PTPRZ1, and ALK expression in human glioblastoma (*GBM*) cell lines and primary lines.** *A*, PTN is expressed in all five established glioblastoma lines and all three primary cultures tested, as determined by Western blot analysis of a 20 μl aliquot of conditioned media from each line and RT-PCR using total RNA as described under "Experimental Procedures." Commercial PTN was used as a positive control (+). Both the expected 18-kDa band and a 15-kDa band species were immunodetected by anti-PTN antibodies. Expression status of PTPRZ1 (*B*) and ALK (*C*) was likewise determined by Western analysis on 20 μg of cell lysate and by RT-PCR. *D*, summary of ALK and PTPRZ1 receptor expression in glioblastoma lines. Cell lines expressing neither receptor, one receptor or the other, or both receptors are represented.

reverse transcribed, second strand synthesis was performed, and *in vitro* transcription was performed with biotinylated nucleotides using the ENZO BioArray High Yield Kit (Enzo Diagnostics, Farmingdale, NY). 15 μg of fragmented cRNA probe was hybridized to Affymetrix HG-U133A arrays (Affymetrix, Santa Clara, CA) and stained with streptavidin-phycoerythrin. Microarrays were scanned in the Genearray Scanner (Hewlett Packard) to a normalized target intensity of 2500. Microarray Suite 5.0 was used to define absent/present calls and generate cel files using the default settings. All grid placements and microarrays were visually reviewed for accuracy and array defects. Data files (cel) were uploaded into the dCHIP program (www.dchip.org/) and normalized to the median intensity array. Quantification was performed using model-based expression and the perfect match minus mismatch method implemented in dCHIP (29).

Patient Tumor Lysates and Tissue Microarray—Protein lysates from frozen tumor samples were prepared as described previously (30), and 30 μg of each sample was run on 15% SDS-PAGE for PTN immunoblotting as described above. The tissue microarray was generated and immunohistochemically stained as described previously (30). Briefly, a tissue array consisting of three representative 0.6-mm cores from formalin-fixed, paraffin-embedded tumor and corresponding normal brain blocks from each of 48 glioblastomas was constructed. Deparaffinized sections were immersed in 0.01 M citrate buffer, pH 6, for 30 min in a pressure cooker for antigen retrieval. Peroxidase activity was quenched with 3% hydrogen peroxide in water, and primary antibodies were diluted (PTN, 1:500; PTPRZ1, 1:100) in normal horse serum (PTPRZ1 and PTN) or normal goat serum (ALK) and applied for 16 h at 4 °C, followed by biotinylated secondary antibodies (Vector) at 1:1000 dilution for 1 h and avidin-biotin complex (Elite ABC; Vector) for 1 h. Negative control slides received normal mouse serum (DAKO) as the primary antibody. Vector NovaRed (Vector) was used to visualize specific antibody localization, and slides were counterstained with Harris hematoxylin. Staining intensity was scored by a neuropathologist (K. Y.) based on a scale of 0–2, in which negatively stained specimens were graded 0, weakly positive samples were graded 1, and strongly positive spots were graded 2.

RESULTS

PTN, PTPRZ1, and ALK Expression—To better understand the mechanisms by which PTN and its receptors contribute to glioblastoma pathogenesis, we assembled a panel of five established glioblastoma cell lines and three low passage primary glioblastoma cultures derived from patient tumors and determined PTN, PTPRZ1, and ALK expression. All eight glioblastoma cell lines expressed and secreted PTN into conditioned media, as detected by RT-PCR and Western blotting (Fig. 1*A*). Interestingly, in addition to the expected 18-kDa PTN band, we also observed a second band migrating at 15 kDa that was immunodetected by anti-PTN antibodies in several cell lines. Two cell lines were strongly positive for PTPRZ1 expression (LN229 and U373MG), whereas the cell lines U87MG,

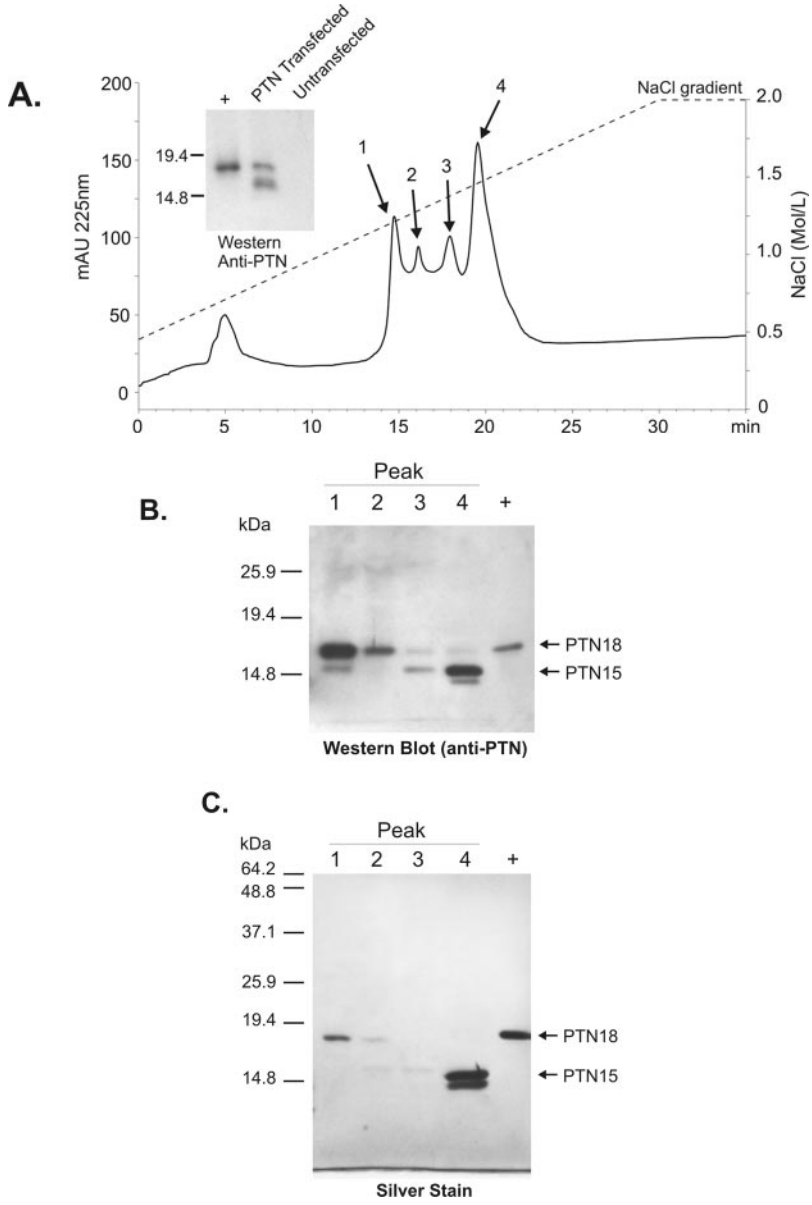

FIG. 2. **Purification of two forms of PTN.** Two forms of recombinant human PTN migrating at 18 and 15 kDa were detected by Western blot in conditioned media of transfected HEK 293T cells (*A*, *inset*). Full-length commercial PTN running at 18 kDa was used as a positive control. PTN was then purified by successive heparin-Sepharose and ion-exchange (sulfopropyl column) HPLC chromatographies. *A*, HPLC chromatogram shows the separation of two major protein peaks (peaks 1 and 4) and two minor peaks (peaks 2 and 3) using a NaCl gradient. *B*, Western blot for PTN on aliquots of each protein peak revealed purification of PTN18 in peaks 1 and 2, and of PTN15 in peaks 3 and 4. *C*, silver staining showed that PTN18 and PTN15 were predominantly recovered in major peaks 1 and 4, respectively, and that the preparations were highly pure. PTN seen in peaks 2 and 3 by Western blot is barely detectable by silver stain and is likely due to carryover from peaks 1 and 4.

GM2313, and GM97 exhibited low to intermediate PTPRZ1 expression (Fig. 1*B*). Similarly, ALK expression was strong in three glioblastoma cell lines (U87MG, U138MG, and GM1600), whereas GM97 demonstrated weaker protein expression (Fig. 1*C*). Altogether, the cells in this panel represent strategic combinations in which to test the role of these receptors in PTN signaling, as summarized in Fig. 1*D*.

Expression and Purification of Recombinant Human PTN from HEK 293T Cells—Because commercially available PTN expressed in baculovirus-infected insect cells lacks mitogenic activity (31) (described by manufacturers; data not shown), we produced recombinant human PTN by transfecting HEK 293T cells with a cloned full-length human PTN cDNA. Like conditioned media from the glioblastoma cell lines, Western blotting showed that two forms of PTN were secreted into HEK 293T conditioned media: one at the expected full-length size of 18 kDa, and the other migrating at 15 kDa (Fig. 2*A*, *inset*). PTN from ~2 liters of transfected cell conditioned media was purified in two successive chromatography steps. Media were first applied onto a heparin-Sepharose column, and then the resulting eluate was further fractionated by cation exchange chromatography using a sulfopropyl column on HPLC. As shown in Fig. 2*A*, elution with a NaCl gradient in HPLC resulted in a chromatogram with two main peaks (peaks 1 and 4) surrounding two minor peaks (peaks 2 and 3). Immunoblotting of the main fractions with an anti-PTN antibody revealed bands with apparent molecular masses of 18 and 15 kDa corresponding to full-length PTN (peak 1) and the smaller form of PTN (peak 4), respectively (Fig. 2*B*). Edman sequencing of these two fractions after SDS-PAGE and polyvinylidene difluoride transfer resulted in identical N-terminal sequences of GKKEKPEKKVKK, further confirming their identities as PTN and suggesting that the 15-kDa form may be C-terminally modified. Silver staining revealed >95% purity in these fractions, as shown in Fig. 2*C*. Analysis of the two minor peaks showed weaker bands with similar molecular masses of 18 kDa (fraction 2) and 15 kDa (fraction 3) by Western blot, and these bands are most likely due to carryover from the main peaks. Because their band intensities were much lower and barely detectable by silver staining, they were not studied any further. PTN from fractions 1 and 4, designated PTN18 and PTN15, respectively, was used in all subsequent experiments.

PTN15 Stimulates Akt and MAPK Activation and Promotes Proliferation in ALK-expressing Glioblastoma Cells—The mitogenic activity of purified PTN18 and PTN15 proteins was tested on serum-starved glioblastoma cells. Whereas stimula-

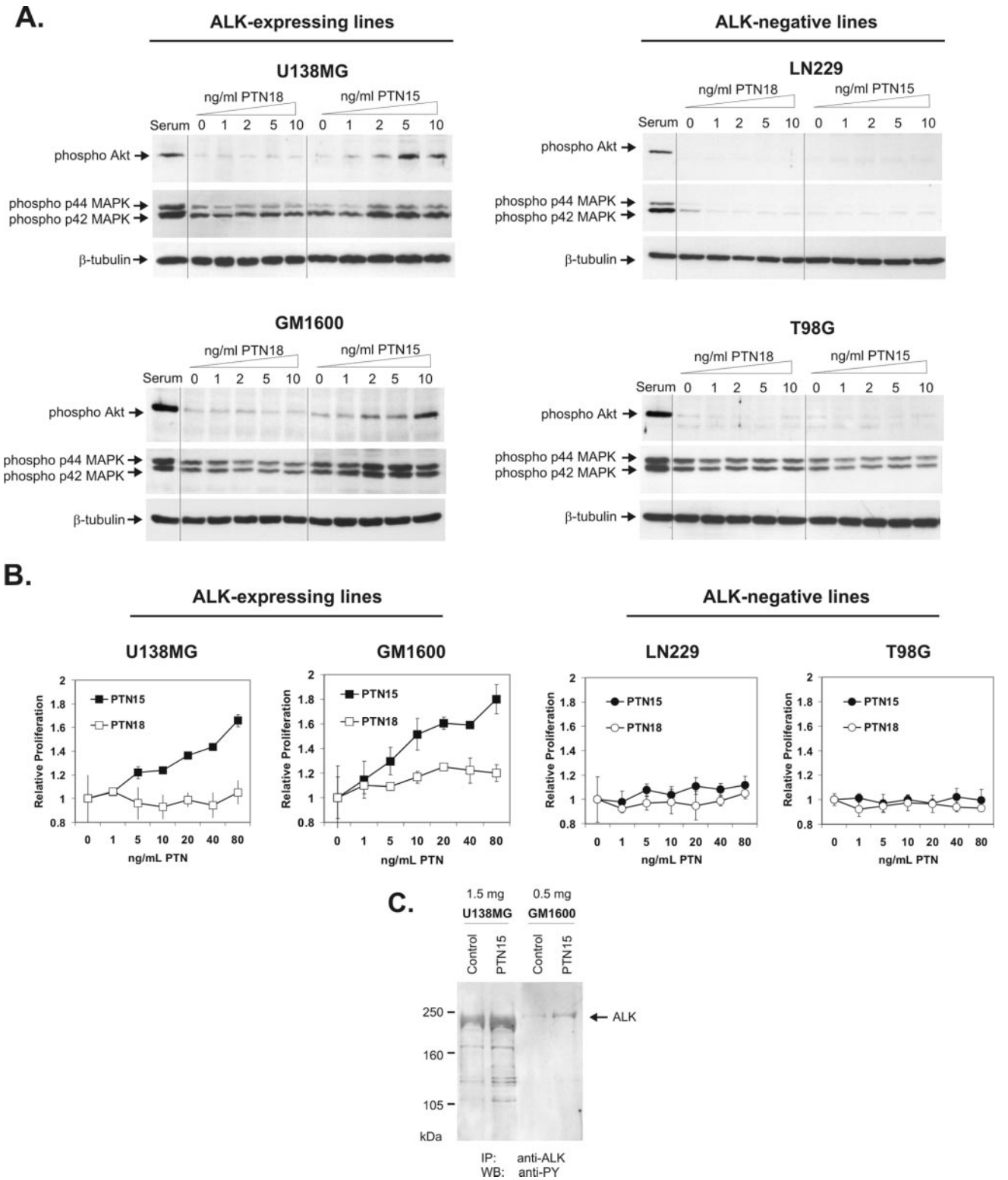

FIG. 3. **Mitogenic effects of PTN15 on ALK-expressing glioblastoma cells.** *A*, glioblastoma lines were serum-starved and stimulated with serum or treated with increasing doses of either PTN18 or PTN15 for 10 min. Representative Western blots demonstrating the effects of PTN stimulation on phosphorylated Akt (Ser^{473}) and phosphorylated p44/42 MAPK (Thr^{202}/Tyr^{204}) in ALK-expressing or non-ALK-expressing glioblastoma cells are shown. PTN18 was not mitogenic in any of the cell lines, whereas PTN15 dose-dependently increased phosphorylation of Akt and p44/42 MAPK only in ALK-expressing cells. Blots were stripped and probed with β-tubulin for loading control. *B*, proliferation of the same glioblastoma lines in response to increasing concentrations of PTN, showing a growth response only from PTN15 in ALK-expressing cells. *C*, protein lysates from ALK-expressing cells (1.5 mg of U138MG and 0.5 mg of GM1600) treated with 10 ng/ml PTN15 for 10 min were subjected to immunoprecipitation with anti-ALK antibodies and subsequent Western blots with anti-phospho-tyrosine antibodies. Tyrosine phosphorylation of ALK is induced in response to PTN15 treatment.

tion of glioblastoma cells with increasing doses of PTN18 did not induce either Akt or MAPK phosphorylation in any of the different cell lines, treatment with PTN15 induced phosphorylation of Akt (Ser^{473}) and MAPK (Thr^{202}/Tyr^{204}) in ALK-expressing glioblastoma cell lines in a dose-dependent manner (Fig. 3*A*). PTN15-induced Akt and MAPK phosphorylation

could be detected after 5 min of treatment and was maintained for at least 30 min, although the activation kinetics were slightly different for each responsive line (data not shown). The signaling activities of PTN15 appeared to be dependent on ALK because each of the responsive cell lines (U138MG and GM1600, Fig. 3*A*; U87MG, data not shown) was ALK-positive (as shown in Fig. 1*C*), whereas none of the lines lacking ALK expression demonstrated Akt or MAPK activation (LN229 and T98G, Fig. 3*A*; U373MG, data not shown). Commercial PTN, which is full length and migrates at 18 kDa like purified PTN18, likewise did not exhibit mitogenic effects on any of the cell lines (data not shown). These results suggest that the mitogenic activity of PTN in glioblastoma cells is primarily dependent on the smaller PTN15 form and not on the full-length PTN18 protein. This is in agreement with a previous study that identified and associated a C-terminally truncated form of PTN with mitogenic activities involving stimulation of phosphatidylinositol 3-kinase and MAPK pathways (24).

In parallel with Akt and MAPK activation studies, the ability of PTN18 and PTN15 to stimulate glioblastoma cell proliferation was also characterized. Similar to the results obtained in pathway activation analyses, varying doses of PTN18 were unable to induce a growth response in any of the cell lines. However, PTN15 was effective in increasing proliferation roughly 2-fold at higher doses in ALK-expressing glioblastoma cells, but not in cells lacking ALK expression (Fig. 3*B*).

To next examine ALK tyrosine phosphorylation in response to PTN 15, U138MG and GM1600 ALK-expressing cells were stimulated with 10 ng/ml PTN15, and their protein lysates were subjected to immunoprecipitation with anti-ALK antibodies followed by Western blot analysis with anti-phospho-tyrosine antibodies. As shown in Fig. 3*C*, a basal level of ALK tyrosine phosphorylation is detectable in control untreated cells; however, ALK tyrosine phosphorylation is markedly increased upon PTN15 treatment. In contrast, PTN18 did not promote tyrosine phosphorylation of ALK in these cell lines (data not shown). Collectively, these results indicate that PTN15 signaling through ALK is specifically responsible for the growth-promoting activities of PTN.

PTN18 Promotes Haptotactic Migration of PTPRZ1-expressing Glioblastoma Cells—PTN has been shown to stimulate migration of embryonic neurons in the developing central nervous system as well as glioma cells through the receptor PTPRZ1 (15, 17). Thus, the ability of PTN18 and PTN15 to induce chemotactic and haptotactic migration was next examined in our panel of glioblastoma cells. For chemotactic migration, soluble PTN was added to medium in the lower chambers of Transwell modified Boyden chambers as a chemoattractant. Using PTN concentrations up to 100 ng/ml, neither PTN18 nor PTN15 exhibited significant chemotactic effects on any of the glioblastoma cell lines (data not shown). However in haptotactic migration assays, in which PTN15 or PTN18 was presented as a substrate coated on the undersides of Transwell filters, three of the glioblastoma cell lines displayed significant migratory responses only to PTN18 coated at 5 μg/ml (Fig. 4). In contrast, migration of these responsive cell lines was identical to background BSA-induced levels when filters were coated with equivalent concentrations of PTN15. Notably, only glioblastoma cells expressing PTPRZ1 exhibited haptotactic migration in response to PTN18 (U87MG, U373MG, and LN229). Cells lacking PTPRZ1 expression did not migrate in response to PTN18 (or PTN15) substrates. In concordance with its intermediate PTPRZ1 expression level, U87MG cells migrated on PTN18 at a lower level than U373MG or LN229 cells, both of which express PTPRZ1 robustly. Coating with commercial PTN revealed the same results as PTN18 (data not shown). These

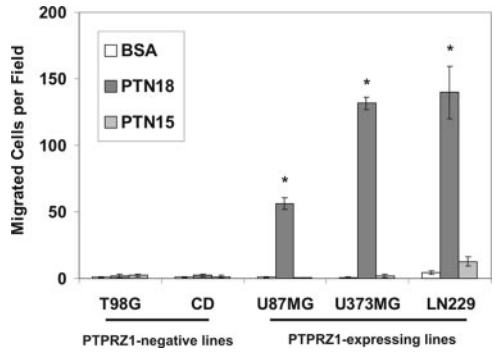

FIG. 4. **Haptotactic migration induced by PTN18 in PTPRZ1-expressing glioblastoma cells.** The undersides of Transwell filters containing 8-μm pores were coated with 5 μg/ml PTN18 or PTN15 or with 50 μg/ml BSA as control. Cells successfully migrating through the pores toward substrate coatings after 6 h were fixed and stained. 5 random fields per filter at ×40 magnification were counted to quantitate cell migration. *, PTN18 promoted statistically significant haptotactic migration only in PTPRZ1-expressing cells ($p < 0.0001$).

findings suggest that induction of glioblastoma cell migration by PTN is predominantly due to the biological activity of the PTN18 form through PTPRZ1, and only when it is present as an immobilized substrate. It also appears that the two forms of PTN each possess distinct biological activities in glioblastoma cells, which function through respectively different receptors.

PTN15 and PTN18 Receptor Binding—To assess whether the two PTN forms only bind exclusively to either ALK or PTPRZ1 to elicit their respective activities, or, alternatively, whether they can bind interchangeably but only signal through one receptor, a series of cross-competition experiments were performed. In one approach, the ability of PTN18 to affect PTN15-mediated pathway activation through ALK was tested. As expected, ALK-expressing cells (U138MG and GM1600) treated only with PTN15 demonstrated an induction of Akt and p44/42 MAPK phosphorylation (Fig. 5*A*). With PTN15 levels held constant, however, the addition of increasing amounts of PTN18 effectively reduced Akt and MAPK phosphorylation in a dose-dependent fashion, suggesting that PTN18 can compete with PTN15 for binding to ALK. In the reverse approach, the ability of PTN15 to affect PTN18-mediated glioblastoma cell migration through PTPRZ1 was also studied. Using Transwell filters coated with a fixed amount of PTN18, the addition of increasing amounts of PTN15 also dose-dependently inhibited migration of the PTPRZ1-expressing cell lines LN229 and U373MG (Fig. 5*B*). These results support a model where the two forms of PTN are capable of binding to both receptors, in which PTN15 only mediates mitogenic signaling through ALK, and PTN18 only stimulates migration through PTPRZ1.

C-terminal Processing of PTN18 Generates PTN15—PTN is not known to be glycosylated, and no evidence of splice variants has been reported. As suggested by others, it is possible that PTN15 is the result of C-terminal processing or truncation because both PTN18 and PTN15 contain identical N-terminal sequences. To further characterize the biochemical differences between the two species, the purified proteins were subjected to MALDI-MS or electrospray LC-MS analyses. The observed molecular mass of PTN18 as determined by electrospray LC-MS matched the theoretical mass of full-length PTN. On the other hand, the PTN15 fraction exhibited an observed mass that was 1456.6 Da smaller than that of full-length PTN18 in both MALDI-MS and LC-MS analyses. This mass difference is concordant with a truncation of the last 12 amino acids from the C terminus of PTN (KKEGKKQEKMLD; theoretical mass difference, 1460.8), suggesting that PTN15 is generated by a C-terminal truncation between Lys^{124} and Lys^{125}.

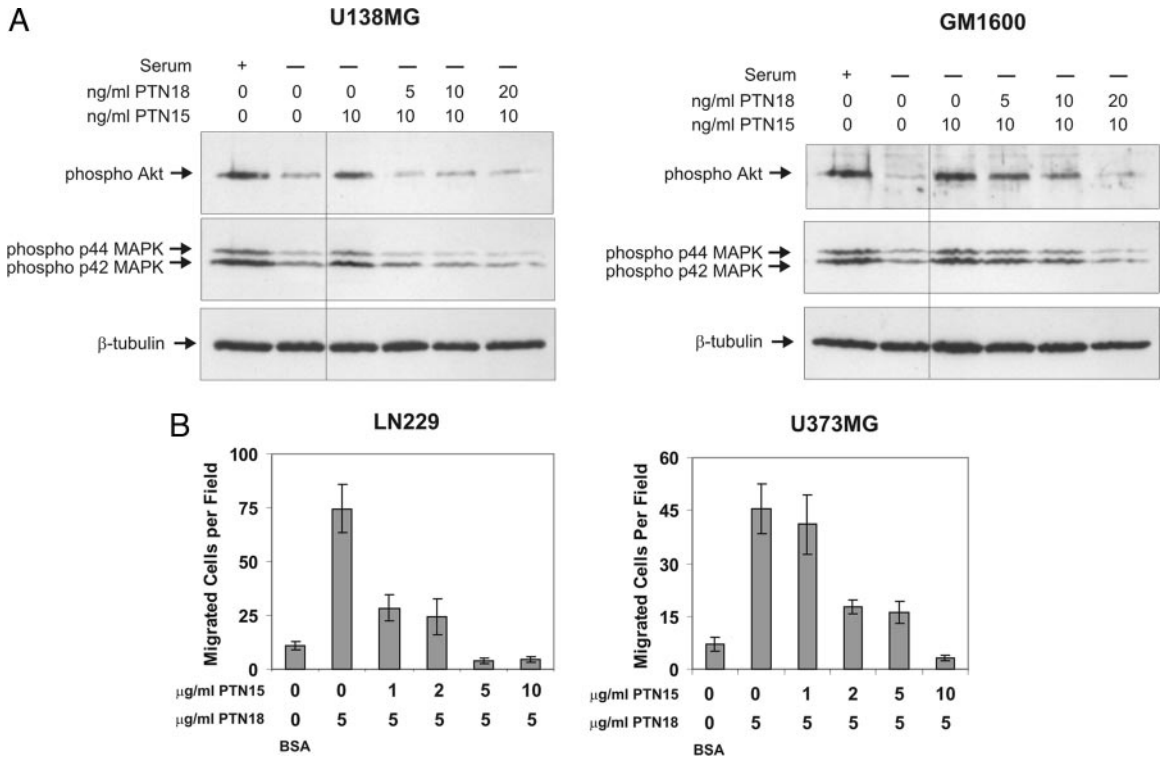

FIG. 5. **Cross-competition of PTN15 and PTN18 for receptor binding and signaling.** *A*, serum-starved ALK-expressing glioblastoma cells (U138MG and GM1600) were treated with serum or stimulated with a constant dose of PTN15 alongside escalating doses of PTN18. Western blot analysis of Akt and p44/42 MAPK phosphorylation shows that the addition of PTN18 inhibits the mitogenic signaling of PTN15, presumably by competing for ALK binding. *B*, haptotactic migration of PTPRZ1-expressing glioblastoma cells (LN229 and U373MG) through PTN18-coated Transwell filters is dimished by the addition of increasing amounts of PTN15, indicating that the two forms of PTN may bind either receptor but only signal through one.

To determine whether the C-terminal truncation in PTN15 was due to alternative splicing of the PTN mRNA, Northern analysis was performed on total RNA isolated from the glioblastoma cells used in this study with a PTN-specific probe, revealing a single ~1.5-kb band in each cell line corresponding to the full-length transcript. Because the resolution of the Northern analysis was likely insufficient to detect a difference potentially as small as 36 bp, RT-PCR with oligo(dT) priming was performed on the same RNA samples using three different pairs of PCR primers flanking the putative truncation site and the 3′-untranslated region. Only one PCR product corresponding to the expected full-length transcript size was obtained for each cell line and each primer set, indicating that PTN15 was not likely the result of mRNA splicing (data not shown).

To further investigate the C-terminal processing of PTN15, a set of constructs was engineered in which a V5-His epitope was added to the C terminus of full-length PTN18 (pcDNA3.1-PTN18-V5), a PTN15 clone missing the last 36 coding bases (translating into a 12-amino acid truncation) was generated (pcDNA3.1-PTN15), and a V5-His epitope was fused to the C terminus of the PTN15 construct (pcDNA3.1-PTN15-V5) (Fig. 6*A*). Along with the native full-length PTN clone (pcDNA3.1-PTN18), these constructs were transfected into HEK 293T cells, and their secreted protein products in conditioned media were analyzed by Western blot. Both the full-length 18-kDa form and processed 15-kDa form were detected by a PTN-specific antibody in the media of cells transfected with pcDNA3.1-PTN18 (Fig. 6*B*, *lane 1*), whereas pcDNA3.1-PTN15 only produced a 15-kDa species (Fig. 6*B*, *lane 2*) that migrated directly alongside the naturally processed PTN15 species seen in *lane 1*. For constructs containing V5-His epitopes fused to their C termini, the PTN protein products migrated at accordingly higher molecular masses, with the tagged full-length construct running slightly higher than its tagged, truncated counterpart, as expected (Fig. 6*B*, *lanes 3* and *4*). Processing of the tagged full-length protein into PTN15 was observed, as seen in *lane 3* of Fig. 6*B*, whereas no further processing was detected in the tagged PTN15 protein. After stripping and reprobing the identical blot with a V5 antibody, no bands were detected in either of the untagged PTN variants as expected (Fig. 6*C*, *lanes 1* and *2*), whereas their tagged counterparts were clearly detected (Fig. 6*C*, *lanes 3* and *4*). Significantly, the processed 15-kDa PTN form, which was detected in pcDNA3.1-PTN18-V5-transfected media when probed with a PTN antibody (Fig. 6*B*, *lane 3*), was not immunodetected with the V5 antibody (Fig. 6*C*, *lane 3*), confirming that a C-terminal cleavage of the full-length protein occurs to generate PTN15.

Recombinantly Engineered PTN15 Demonstrates the Same Biological Activities as Naturally Produced PTN15—To confirm that the engineered PTN15 retained the biological activity of its naturally processed counterpart, both wild type and truncated constructs were expressed, and the resulting conditioned media were collected for PTN protein purification as described above. As shown in Fig. 7*A*, ion-exchange HPLC separation of recombinant protein expressed from pcDNA3.1-PTN15 (designated ePTN15 for engineered PTN15) revealed a single peak (*dotted chromatogram*) that exhibited the same chromatographic profile and retention time as PTN15 naturally processed from the full-length protein (*solid chromatogram*). Silver staining (data not shown) and Western blotting of the purified ePTN15 peak with an anti-PTN antibody (Fig. 7*B*) confirmed its purity and identity as a 15-kDa PTN species. Subsequently, the biological activity of ePTN15 in glioblastoma cells was compared against commercial PTN, purified PTN18, and its naturally produced PTN15 derivative from HEK 293T preparations. Whereas commercial PTN and PTN18 were un-

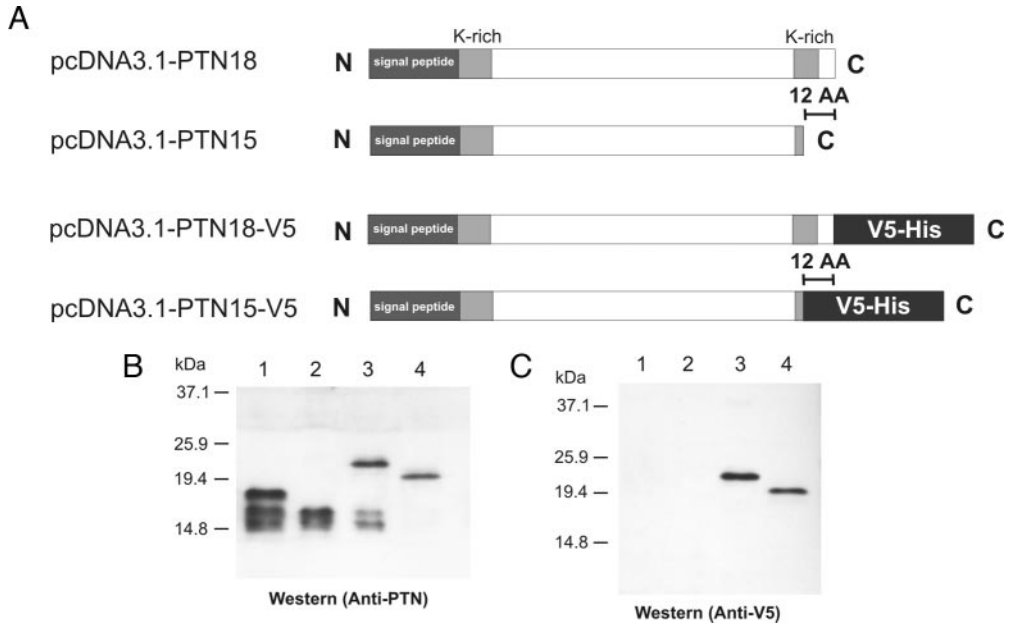

FIG. 6. **Post-translational C-terminal processing of PTN.** *A*, full-length PTN cDNA (pcDNA3.1-PTN18) was fused in-frame with a C-terminal V5-His epitope tag (pcDNA3.1-PTN18-V5) after removal of the endogenous stop codon. A truncated PTN clone was generated by inserting a stop codon 12 codons upstream of the endogenous stop codon (pcDNA3.1-PTN15). This artificial stop codon was likewise removed from the truncated clone and fused in-frame with V5-His at the C terminus (pcDNA3.1-PTN15-V5). *B*, Western blot detection of PTN species secreted by HEK 293T cells transfected with the various PTN constructs using an anti-PTN antibody (pcDNA3.1-PTN18, *lane 1*; pcDNA3.1-PTN15, *lane 2*; pcDNA3.1-PTN18-V5, *lane 3*; pcDNA3.1-PTN15-V5, *lane 4*). *C*, identical blot as described in *B* after stripping and reprobing with anti-V5 antibody. The absence of the V5 epitope in the PTN15 species produced from pcDNA3.1-PTN18-V5 indicates that it is post-translationally cleaved at the C terminus (*B* and *C*, *lane 3*).

able to stimulate pathway activation in ALK-expressing glioblastoma cells, both PTN15 and ePTN15 induced Akt phosphorylation in these cells as shown in Fig. 7*C*. Induction of p44/42 MAPK phosphorylation was likewise observed when U138MG cells were treated with PTN15 and ePTN15, whereas in U87MG cells, which contain very high basal levels of MAPK activation, an increase in MAPK activation was not detectable (data not shown), in agreement with a previous report (13). Correspondingly, ePTN15 (but not PTN18) was capable of stimulating proliferation in ALK-expressing cells (Fig. 7*E*). As expected, none of the PTN forms was capable of stimulating Akt phosphorylation or cell growth in ALK-negative cells (Fig. 7, *D* and *E*). Furthermore, in concordance with results described above, ePTN15 did not support haptotactic migration of PTPRZ1-expressing glioblastoma cells like PTN18 did (Fig. 7*F*), thus duplicating the biological activities of naturally processed and purified PTN15.

Expression of PTN, PTPRZ1, and ALK in Glioblastoma Patient Tissues—In a previous study using Affymetrix U95Av2 microarrays to identify molecularly distinct subsets of microscopically identical glioblastomas, we found that PTN and PTPRZ1 were highly expressed in a subset of glioblastomas (27). In a subsequent study in which gene expression profiles of 63 glioblastomas were compared with those of 20 normal brain samples using U133 arrays (28), we consistently observed that PTN and PTPRZ1 mRNA were up-regulated in glioblastomas compared with normal brain by 2.62- and 2.97-fold ($p < 0.0001$ for both), respectively. Significant differential expression of ALK was not detected in these analyses.

Because the two different forms of PTN we observed appeared to confer distinct tumor-promoting activities through respectively different receptors in glioblastoma cells, the protein expression of each species in patient tumors was next examined. Western blot analysis of lysates from frozen tumor and contralateral normal brain specimens from eight glioblastoma autopsy cases revealed that both PTN18 and PTN15 species are detected at roughly equivalent levels and that overall PTN expression is significantly higher in tumor compared with normal brain (Fig. 8*A*). To further profile PTN, PTPRZ1, and ALK protein expression in patient tissues, a tissue microarray consisting of tumor cores and matching normal brain counterparts from 48 glioblastoma patients was constructed and immunohistochemically stained. Mean staining intensities of PTN, PTPRZ1, and ALK on tumor or normal brain TMA spots are shown in Fig. 8*B*, whereas representative stained tissue spots are shown in Fig. 8*C*. Similar to expression data obtained from microarray, PTN and PTPRZ1 staining were both significantly stronger in glioblastoma compared with normal brain ($p = 0.0005$ and $p < 0.0001$, respectively), whereas mean staining intensity of ALK was not significantly different between tumor and normal tissue.

DISCUSSION

Recent advances in the understanding of brain tumors have suggested that many of the mechanisms involved in normal nervous system development are inappropriately reiterated in brain cancer. Following this notion, we and others have discovered that expression of PTN and its receptor PTPRZ1, both of which are developmentally regulated in the nervous system, is increased in glioblastoma patient tumors compared with normal brain. In the present study, we have isolated and characterized two forms of PTN secreted from human cells, each demonstrating distinct signaling capabilities through the receptors ALK and PTPRZ1, through which each can independently mediate either tumor cell growth or migration. To our knowledge, this is the first report in which the signaling effects of PTN through both ALK and PTPRZ1 have been studied together. Our data indicate that the associated functions of the ALK and PTPRZ1 pathways are stimulated by different forms of PTN, an observation that may partially clarify some of the contradictory results regarding its biological activities. Because these pathways appear to be highly activated in glioblas-

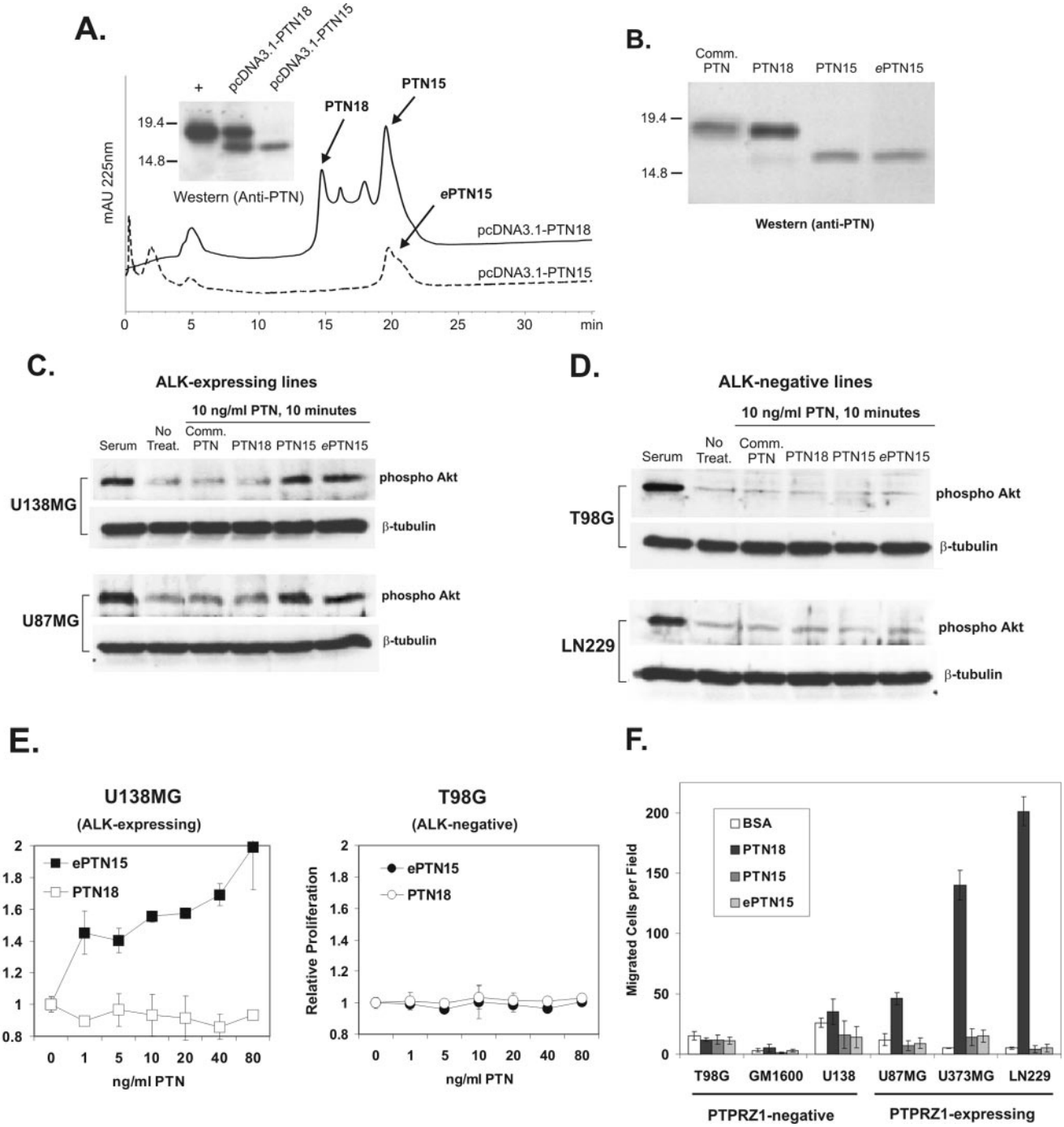

FIG. 7. **Recombinantly generated ePTN15 demonstrates identical biological activities as naturally produced PTN15.** PTN secreted into conditioned media of HEK 293T cells transfected with either pcDNA3.1-PTN18 or pcDNA3.1-PTN15 (*A*, *inset*) was purified as described in the Fig. 2 legend and under "Experimental Procedures." *A*, ePTN15 expressed from the truncated pcDNA3.1-PTN15 clone (*dotted chromatogram*) exhibited the same chromatographic profile as naturally processed PTN15 (*solid chromatogram*) after HPLC separation. *B*, Western blot analysis of purified PTN18, PTN15, and ePTN15 peaks. *C* and *D*, Akt phosphorylation (Ser^{473}) in ALK-expressing (*C*) or ALK-negative (*D*) glioblastoma cells after treatment of serum-starved glioblastoma cells with serum-containing medium, 10 ng/ml commercial PTN, or purified PTN18, PTN15, or ePTN15 for 10 min, as shown by Western blot. PTN15 and ePTN15 both display mitogenic activity in ALK-expressing cells. β-Tubulin is shown as a loading control. *E*, cell proliferation in response to increasing doses of PTN18 or ePTN15 shows ALK-dependent mitogenic activity of ePTN15. *F*, haptotaxis assays on BSA, PTN18, PTN15, or ePTN15 substrates revealed PTPRZ1-dependent migration only on PTN18.

toma, they may also represent promising therapeutic targets.

Although several groups have described a lack of growth response when glioblastoma cells were treated with PTN (10, 11), these studies did not establish ALK expression status and utilized commercially produced PTN that may have been mitogenically inactive. In the current study, we observed that ALK expression correlated with Akt and MAPK activation, as well as stimulation of cell proliferation, when glioblastoma cells were treated with PTN15, but not PTN18. Not only does this support data suggesting that C-terminally processed (but not full-length) PTN expressed in human cells is mitogenically active (24), it further substantiates the critical role of ALK as the mitogenic receptor for PTN (32) because glioblastoma cells lacking ALK expression were unresponsive to PTN15. Interestingly, full-length PTN (with an intact C terminus) has previously been reported to possess mitogenic and transforming

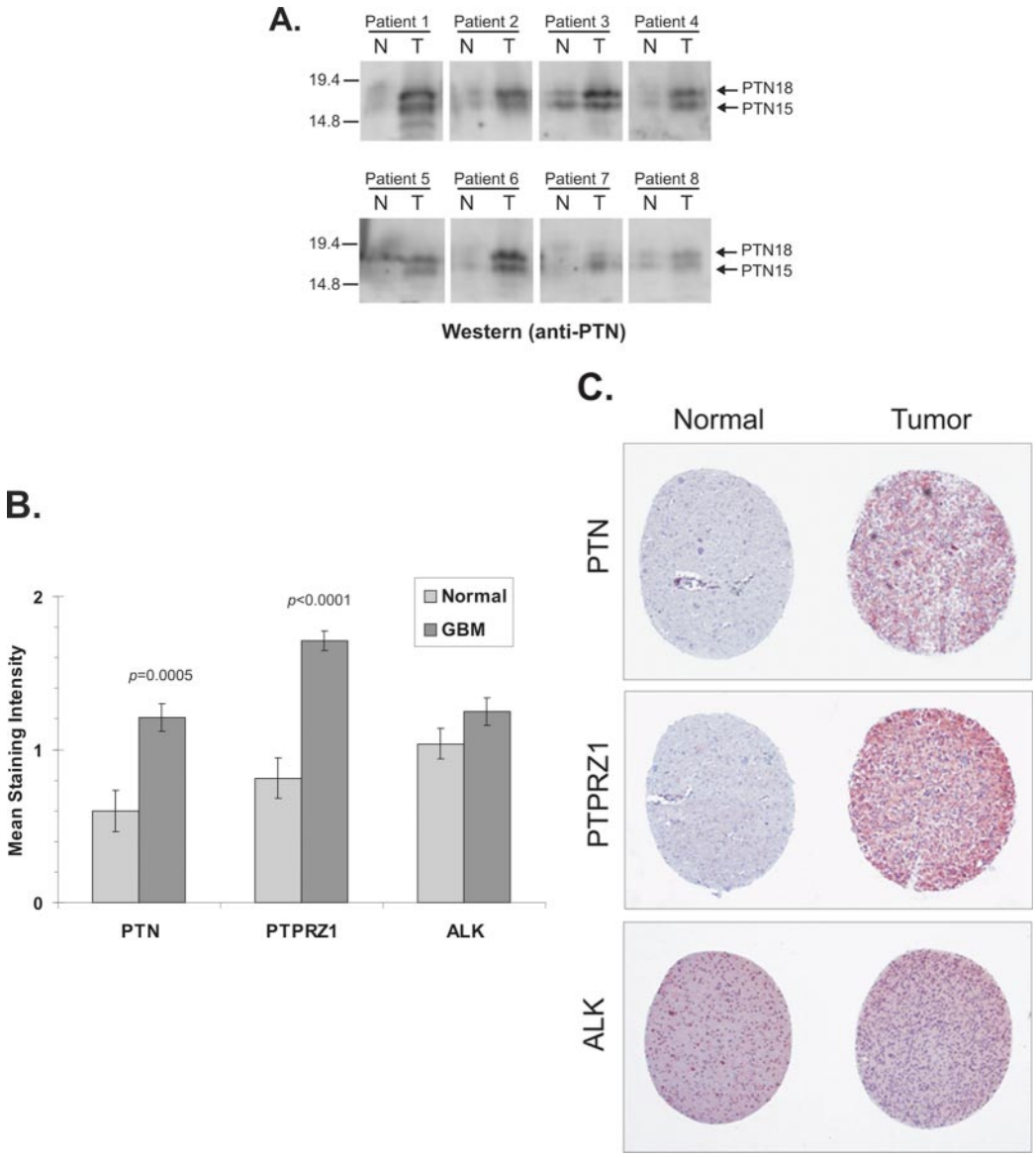

FIG. 8. **Expression of PTN, PTPRZ1, and ALK in glioblastoma patients.** *A*, protein lysates prepared from frozen tumor specimens (*T*) or contralateral normal brain (*N*) of eight glioblastoma patients after autopsy were run on SDS-PAGE and analyzed for PTN expression by Western blot with an anti-PTN antibody. Both PTN18 and PTN15 are seen at significantly higher levels in tumors than in normal brain. *B*, mean immunohistochemical staining intensity of PTN, PTPRZ1, and ALK in 48 glioblastomas or corresponding normal brain spots as represented on a tissue microarray. Staining intensity was scored on a scale of 0–2 as described under "Experimental Procedures." PTN and PTPRZ1 staining were significantly higher in glioblastoma compared with normal brain, whereas no significant difference in ALK was detected. *C*, representative immunohistochemical staining of normal and tumor tissue microarray spots for PTN, PTPRZ1, and ALK.

activity (33, 34), however, these studies utilized NIH 3T3 and CHO-K1 mouse cells to express PTN. In this work and another study (24) in which mitogenic activity was exclusive to a smaller form of PTN, the protein was expressed in human cells. As has been speculated before, the disparities in mitogenic activity from various PTN preparations could be due to differences in protein folding, modifications, and even the type of mammalian expression system used (33). We found that PTN15 stimulated ALK-expressing glioblastoma cells, whereas purified PTN18 and commercially available PTN were unable to stimulate Akt or MAPK phosphorylation in any of our glioblastoma cell lines.

In contrast to the functional effects of PTN15, PTN18 induced migration of PTPRZ1-expressing glioblastoma cells when used as a haptotactic substrate. Similar to the requirement of ALK expression for PTN15-induced mitogenic effects, a tight correlation between PTPRZ1 expression and haptotactic migration on PTN18 was observed because glioblastoma cells lacking PTPRZ1 did not demonstrate increased migration. Although ALK expression did not appear to be a factor in the migration phenotype, we cannot exclude the possibility that another PTN receptor, syndecan-3, may play a role in mediating PTN18-induced haptotactic migration in some glioblastoma cells, as previously suggested (17, 35). The downstream signaling components involved in PTN18-PTPRZ1-mediated cell migration are still unclear. One possibility is raised by a recent study showing the preservation of tyrosine-phosphorylated β-catenin due to inhibition of PTPRZ1 phosphatase activity upon PTN binding in U373MG glioblastoma cells (36) because accumulation of tyrosine-phosphorylated β-catenin has been correlated with increased cell migration in a number of studies (37, 38). Whereas we have evidence showing that PTN15 does not affect tyrosine phosphorylation of β-catenin through PTPRZ1,[2] it remains to be seen whether this pathway contributes

[2] K. V. Lu, K. A. Jong, and P. S. Mischel, unpublished data.

to or is required for PTN18-induced migration. Other clues to the signaling pathways involved in PTPRZ1-dependent haptotactic migration on PTN18 come from studies on midkine, a closely related homologue of PTN, in which midkine immobilized on polystyrene beads induced PTPRZ1-dependent phosphatidylinositol 3-kinase and MAPK activation, which appeared to be necessary for haptotactic migration (39). However, a parallel mechanism for PTN using PTN18 coated beads has not yet been investigated.

Based on these collective results and especially those of U87MG cells that express both ALK and PTPRZ1, it appears that the two different forms of PTN mediate distinct functional effects through different cell surface receptors. PTN15 stimulation through ALK activates a mitogenic signal transduction pathway, whereas binding of immobilized PTN18 to PTPRZ1 induces cell migration. Although we were interested in discovering whether the downstream pathways of these two PTN receptors merged synergistically to promote glioblastoma progression, our data suggest that they probably represent independent pathways leading to dissimilar effects. Whereas it is conceivable that each pathway may independently activate some of the same signaling molecules, as of now there is no evidence suggesting that the two affect each other's functions.

Our characterization of a 12-amino acid truncation at the C terminus of PTN15 is supported by the fact that recombinantly engineered ePTN15 demonstrated identical PAGE migration and chromatographic separation as naturally processed PTN15, and more importantly, it possessed the biological activities of naturally processed PTN15. Based on our data from C-terminal epitope-tagged PTN variants, as well as Northern and RT-PCR analyses, PTN15 is most likely generated by post-translational C-terminal cleavage. It is still unclear what protease or mechanism generates PTN15. Due to the lysine-rich nature of PTN and the fact that the 12-amino acid truncation occurs in the middle of a series of lysine residues at the C terminus, it is possible that this small structural difference may be significant enough to alter the physical interactions between the two forms of PTN and their receptors, thus modulating their capability to activate signal transduction. The functional data obtained from our receptor binding competition assays suggest that the two forms of PTN can bind to both ALK and PTPRZ1, but with PTN15 only capable of eliciting signaling properties through ALK and PTN18 only capable of eliciting signaling properties through PTPRZ1, respectively. In addition, because PTN is thought to dimerize (40), and mutant PTN variants have been shown to be dominant negative effectors of wild type PTN (41), it is possible that the two PTN forms can affect each other's functions or receptor binding characteristics by forming inactive heterodimers. This could explain why various laboratories using different preparations of PTN have reported partially contradictory results regarding its biological activities.

Whereas expression of both PTN and PTPRZ1 was higher in glioblastomas compared with normal brain as demonstrated by DNA microarray and tissue microarray, we did not observe a significant difference in ALK expression in these analyses. This may suggest a more prominent role for the PTN-PTPRZ1 migration pathway in glioblastoma biology. However, the presence of PTN15 at clearly higher levels in tumors compared with normal brain could still render autocrine and even paracrine growth advantages to glioblastomas and their surrounding cells. For example, glioblastomas are highly vascularized tumors, and PTN has been shown to stimulate a variety of endothelial cell types, including cerebral microvascular endothelial cells (17, 42). Therefore PTN, PTPRZ1, and ALK may represent promising targets for glioblastoma therapy. Moreover, such therapies could address many different pathologic aspects of the disease because of the wide range of activities of PTN. Indeed, silencing of PTN expression has already shown it to be rate-limiting for tumor growth in animal models (43), whereas knockdown of ALK likewise reduced tumor size in glioblastoma xenografts (13), and blocking of PTPRZ1 inhibited migration of rat glioma cells (17).

In summary, we have purified and characterized two forms of PTN that mediate either mitogenic activity through ALK or migration through PTPRZ1. Because the expression of PTN and its receptor PTPRZ1 is significantly higher in human glioblastoma patients compared with normal brain, PTN and its receptor-associated pathways may represent potentially effective therapeutic targets for both the proliferative and invasive characteristics of glioblastoma.

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REFERENCES

1. Mischel, P. S., and Cloughesy, T. F. (2003) *Brain Pathol.* **13,** 52–61
2. Bloch, B., Normand, E., Kovesdi, I., and Bohlen, P. (1992) *Brain Res. Dev. Brain Res.* **70,** 267–278
3. Li, Y. S., Milner, P. G., Chauhan, A. K., Watson, M. A., Hoffman, R. M., Kodner, C. M., Milbrandt, J., and Deuel, T. F. (1990) *Science* **250,** 1690–1694
4. Merenmies, J., and Rauvala, H. (1990) *J. Biol. Chem.* **265,** 16721–16724
5. Silos-Santiago, I., Yeh, H. J., Gurrieri, M. A., Guillerman, R. P., Li, Y. S., Wolf, J., Snider, W., and Deuel, T. F. (1996) *J. Neurobiol.* **31,** 283–296
6. Fang, W., Hartmann, N., Chow, D. T., Riegel, A. T., and Wellstein, A. (1992) *J. Biol. Chem.* **267,** 25889–25897
7. Klomp, H. J., Zernial, O., Flachmann, S., Wellstein, A., and Juhl, H. (2002) *Clin. Cancer Res.* **8,** 823–827
8. Vacherot, F., Caruelle, D., Chopin, D., Gil-Diez, S., Barritault, D., Caruelle, J. P., and Courty, J. (1999) *Prostate* **38,** 126–136
9. Wellstein, A., Fang, W. J., Khatri, A., Lu, Y., Swain, S. S., Dickson, R. B., Sasse, J., Riegel, A. T., and Lippman, M. E. (1992) *J. Biol. Chem.* **267,** 2582–2587
10. Mentlein, R., and Held-Feindt, J. (2002) *J. Neurochem.* **83,** 747–753
11. Muller, S., Kunkel, P., Lamszus, K., Ulbricht, U., Lorente, G. A., Nelson, A. M., von Schack, D., Chin, D. J., Lohr, S. C., Westphal, M., and Melcher, T. (2003) *Oncogene* **22,** 6661–6668
12. Deuel, T. F., Zhang, N., Yeh, H. J., Silos-Santiago, I., and Wang, Z. Y. (2002) *Arch. Biochem. Biophys.* **397,** 162–171
13. Powers, C., Aigner, A., Stoica, G. E., McDonnell, K., and Wellstein, A. (2002) *J. Biol. Chem.* **277,** 14153–14158
14. Stoica, G. E., Kuo, A., Aigner, A., Sunitha, I., Souttou, B., Malerczyk, C., Caughey, D. J., Wen, D., Karavanov, A., Riegel, A. T., and Wellstein, A. (2001) *J. Biol. Chem.* **276,** 16772–16779
15. Maeda, N., and Noda, M. (1998) *J. Cell Biol.* **142,** 203–216
16. Maeda, N., Nishiwaki, T., Shintani, T., Hamanaka, H., and Noda, M. (1996) *J. Biol. Chem.* **271,** 21446–21452
17. Ulbricht, U., Brockmann, M. A., Aigner, A., Eckerich, C., Muller, S., Fillbrandt, R., Westphal, M., and Lamszus, K. (2003) *J. Neuropathol. Exp. Neurol.* **62,** 1265–1275
18. Morris, S. W., Naeve, C., Mathew, P., James, P. L., Kirstein, M. N., Cui, X., and Witte, D. P. (1997) *Oncogene* **14,** 2175–2188
19. Iwahara, T., Fujimoto, J., Wen, D., Cupples, R., Bucay, N., Arakawa, T., Mori, S., Ratzkin, B., and Yamamoto, T. (1997) *Oncogene* **14,** 439–449
20. Krueger, N. X., and Saito, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7417–7421
21. Levy, J. B., Canoll, P. D., Silvennoinen, O., Barnea, G., Morse, B., Honegger, A. M., Huang, J. T., Cannizzaro, L. A., Park, S. H., and Druck, T. (1993) *J. Biol. Chem.* **268,** 10573–10581
22. Wang, Y., Zhu, S., Cloughesy, T. F., Liau, L. M., and Mischel, P. S. (2004) *Oncogene* **23,** 1283–1290
23. Lu, K. V., Jong, K. A., Rajasekaran, A. K., Cloughesy, T. F., and Mischel, P. S. (2004) *Lab. Investig.* **84,** 8–20
24. Souttou, B., Ahmad, S., Riegel, A. T., and Wellstein, A. (1997) *J. Biol. Chem.* **272,** 19588–19593
25. Lu, K. V., Rohde, M. F., Thomason, A. R., Kenney, W. C., and Lu, H. S. (1995) *Biochem. J.* **309,** Pt 2, 411–417
26. Jones, M. D., Patterson, S. D., and Lu, H. S. (1998) *Anal. Chem.* **70,** 136–143
27. Mischel, P. S., Shai, R., Shi, T., Horvath, S., Lu, K. V., Choe, G., Seligson, D., Kremen, T. J., Palotie, A., Liau, L. M., Cloughesy, T. F., and Nelson, S. F. (2003) *Oncogene* **22,** 2361–2373
28. Freije, W. A., Castro-Vargas, F. E., Fang, Z., Horvath, S., Cloughesy, T., Liau, L. M., Mischel, P. S., and Nelson, S. F. (2004) *Cancer Res.* **64,** 6503–6510
29. Li, C., and Wong, W. H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 31–36
30. Choe, G., Horvath, S., Cloughesy, T. F., Crosby, K., Seligson, D., Palotie, A., Inge, L., Smith, B. L., Sawyers, C. L., and Mischel, P. S. (2003) *Cancer Res.* **63,** 2742–2746
31. Raulo, E., Julkunen, I., Merenmies, J., Pihlaskari, R., and Rauvala, H. (1992) *J. Biol. Chem.* **267,** 11408–11416
32. Bowden, E. T., Stoica, G. E., and Wellstein, A. (2002) *J. Biol. Chem.* **277,** 35862–35868
33. Bernard-Pierrot, I., Delbe, J., Caruelle, D., Barritault, D., Courty, J., and

Milhiet, P. E. (2001) *J. Biol. Chem.* **276,** 12228–12234
34. Zhang, N., Zhong, R., and Deuel, T. F. (1999) *J. Biol. Chem.* **274,** 12959–12962
35. Raulo, E., Chernousov, M. A., Carey, D. J., Nolo, R., and Rauvala, H. (1994) *J. Biol. Chem.* **269,** 12999–13004
36. Meng, K., Rodriguez-Pena, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M., and Deuel, T. F. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2603–2608
37. Wadham, C., Gamble, J. R., Vadas, M. A., and Khew-Goodall, Y. (2003) *Mol. Biol. Cell* **14,** 2520–2529
38. Muller, T., Choidas, A., Reichmann, E., and Ullrich, A. (1999) *J. Biol. Chem.* **274,** 10173–10183
39. Qi, M., Ikematsu, S., Maeda, N., Ichihara-Tanaka, K., Sakuma, S., Noda, M., Muramatsu, T., and Kadomatsu, K. (2001) *J. Biol. Chem.* **276,** 15868–15875
40. Bernard-Pierrot, I., Heroult, M., Lemaitre, G., Barritault, D., Courty, J., and Milhiet, P. E. (1999) *Biochem. Biophys. Res. Commun.* **266,** 437–442
41. Bernard-Pierrot, I., Delbe, J., Rouet, V., Vigny, M., Kerros, M. E., Caruelle, D., Raulais, D., Barritault, D., Courty, J., and Milhiet, P. E. (2002) *J. Biol. Chem.* **277,** 32071–32077
42. Courty, J., Dauchel, M. C., Caruelle, D., Perderiset, M., and Barritault, D. (1991) *Biochem. Biophys. Res. Commun.* **180,** 145–151
43. Malerczyk, C., Schulte, A. M., Czubayko, F., Bellon, L., Macejak, D., Riegel, A. T., and Wellstein, A. (2005) *Gene Ther.* **12,** 339–346