

# LARGE-SCALE MAMMALIAN EXPRESSION AND PURIFICATION OF PROTEIN TYROSINE PHOSPHATASE PTPBR7

SUJAY TURUVEKERE MALLIKARJUNA, STEFAN E. SZEDLACSEK\*

*Department of Enzymology, Institute of Biochemistry of the Romanian Academy,  
Splaiul Independentei 296, 060031 Bucharest, Romania*

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Protein tyrosine phosphatase PTPBR7 is a receptor-like, transmembrane protein encoded by the mouse *Ptprr* gene, which is restrictedly expressed on the surface of neuronal cells. PTPBR7-deficient mice have been shown to display motor coordination defects. The extracellular domain of PTPBR7 binds to the highly myelinated regions in mouse brains. Identification of associating proteins suggests that PTPBR7 plays a role in cell-cell adhesion and in cerebellar calcium ion homeostasis. The extracellular region of PTPBR7 does not reveal folding similarities to any known subdomain, the structure of this region remaining to be solved. In this study we report large-scale mammalian expression of the ecto domain of PTPBR7. Large-scale expression of the entire extracellular region showed that the protein was unstable during expression and purification, and was prone to proteolytic cleavage. N-terminal sequencing revealed at least 3 major protein cleavage sites in the proximity of the N-terminus of this protein. Shorter constructs were obtained starting from the identified sites. These constructs were resistant to proteolytic cleavage and were expressed in large scale and purified to homogeneity by immobilized metal affinity chromatography and size exclusion chromatography. These purified proteins can be used for further structural and functional *in vitro* studies.

**Key words:** PTPBR7, ecto domain, mammalian expression, protein purification.

## INTRODUCTION

The protein tyrosine phosphorylation is important in various physiological functions of the eukaryotic cells, being involved in many functions such as cell proliferation, survival, migration and differentiation. The Protein Tyrosine Kinases (PTKs) and Protein Tyrosine Phosphatases (PTPs) play opposite roles in tight regulation of protein phosphorylation in the cells (1). Receptor Protein Tyrosine Phosphatases (RPTPs) belong to classical protein tyrosine phosphatases and show strong expression in central and peripheral nervous system. Many RPTPs are

\*Corresponding author: (E-mail: stefan@biochim.ro; Tel: +40-21-2239069; Fax: +40-21-2239068).

involved in axogenesis, synaptogenesis, target contact and plasticity (2, 3). Several *in vivo* roles of RPTPs in neural development and function have been demonstrated (1, 3). Although numerous effectors of the downstream RPTP signaling are known, the information about the ligands of RPTPs is limited (4).

The mouse *Ptprr* gene encodes PTPBR7, a receptor-type isoform. PTPBR7 is the longest of the four isoforms, containing 656 amino acids (5). *Ptprr* belongs to the R7 subgroup of RPTPs composed of a short extracellular region, a transmembrane region, a cytosolic region containing the Kinase Interacting Motif (KIM), and a phosphatase domain (6). PTPBR7 is expressed during early embryogenesis in spinal ganglia and Purkinje cells. After birth, the expression of PTPBR7 occurs in all regions of the brain, whereas it is decreased in maturing Purkinje cells (7). PTPBR7 forms homo-oligomers located at the cell surface, and shows reduced activity in comparison with the cytosolic isoforms that are monomeric (8). All the *Ptprr* isoforms contain Kinase Interacting Motif (KIM), and are known to bind and inactivate Mitogen-Activated Protein Kinases (MAPKs) (5). *Ptprr* knockout mice performed poorly in various locomotive tests, but they show no brain malformation (9). Impairment in cerebellar calcium ion homeostasis is also known to cause ataxia in animal models, similar to *Ptprr* knockout mice (10). Highly myelinated areas in the brain have been recently proved to be sites for PTPBR7 ligands (11). Identified PTPBR7 ligand candidates suggest/indicate the involvement of this receptor in cell-cell adhesion complex during cerebellar development and calcium ion regulated events that are important in neuronal development and plasticity (11).

## MATERIAL AND METHODS

### CLONING OF PTPBR7 CONSTRUCTS

The gene coding for the full PTPBR7 extracellular region cloned into pHLsec mammalian expression vector was a kind gift from Dr Hendriks (11). The pHLsec vector contains a signal sequence for secretion of the protein fused with a C-terminal 6X His-tag. The shorter constructs of PTPBR7 extracellular region were cloned into pHLsec vector at *AgeI* and *KpnI* restriction sites. The plasmid containing full-length extracellular region of PTPBR7 was used as template and the following primers were used: BR7S1 forward, 5'-GAAACCGGTAGTTGGAAGCCGGTGTTTCATTTATGACC-3'; BR7S2 forward, 5'-GAAACCGGTAGCCTGGACATCGCACAAAGAGGC-3'; BR7S3 forward, 5'-GAAACCGGTCATAACTACCACTCCCCTTCCGAAAG-3'; BR7S reverse, 5'-CTTGGTACCCTGCCCTTGTAACCTTTTCTCAAGGGG-3' for the PCR amplification. The PCR product and the pHLsec vector were restriction digested with *AgeI* and *KpnI* enzymes and ligated to obtain desired constructs. The pHLsec vector with RPTP $\mu$  coding gene inserted between *AgeI* and *KpnI* restriction sites was a kind gift from Dr. Aricescu (12).

#### CELL CULTURE AND TRANSIENT TRANSFECTION

HEK293T cells and N-acetylglucosaminyl transferase I-negative 293S GnTII<sup>-</sup> cells (13), unable to synthesize complex N-glycans, a kind gift from Dr. Aricescu, were used for the expression of recombinant PTPBR7 constructs. HEK293T and 293S cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM high glucose, Sigma) supplemented with L-glutamine, non-essential amino acids (Gibco) and 10% fetal calf serum (FCS, Sigma). The cells were maintained in standard flask in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Small-scale transfections were carried out in 6-well plates, while the large-scale cultures for protein productions were performed using expanded-surface polystyrene roller bottles (2125 cm<sup>2</sup>, Greiner Bio-One). Cells were transfected when the confluence of the adherent cells reached about 90%. PTPBR7 constructs purified with Endotoxin-Free Plasmid Mega Kit (Qiagen) were used for transfection with PEI (1:1.5 v:v). After reaching 90% confluence, the cells were transferred into a lower (2%) serum containing media, and the transfection mixture containing DNA-PEI complex was added. HEK293T and 293S cells were grown for 2-3 days and 4-5 days, respectively, for protein expression (12).

#### PTPBR7 DETECTION BY WESTERN BLOTTING

Small aliquots of conditioned media 2-3 days post transfection were separated by SDS-PAGE and then transferred to Immobilon P membrane (Millipore) for 1 h at 75 mA, using a semidry transfer system. The membrane was treated with blocking agent (5% low fat skim milk (Fluka)) for 1 h at room temperature with blocking buffer in a 50 mM Tris, pH 8.0, 150 mM NaCl buffer). Then, the membrane was incubated with PentaHis monoclonal (against 6XHis tag) primary antibody (1:1000 dilution, Qiagen) for 1 h at room temperature, followed by incubation with the goat anti-mouse IgG peroxidase-conjugated secondary antibody (1:2000, Sigma) for 1 h at room temperature. The blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

#### PTPBR7 PROTEIN PURIFICATION

The conditioned media were collected 3 days post transfection in case of HEK293T cells and 5 days in case of 293S cells. Conditioned media were filtered through a 0.2 µm membrane (Express filter, Millipore) and diluted three folds using PBS at pH 8.0 adjusted with Tris buffer. Immobilized metal affinity chromatography (IMAC) purification is performed using nickel-coated chelating Sepharose (GE Healthcare). Beads equilibrated with Phosphate Buffer Saline (PBS) were added to the filtered, diluted conditioned media and incubated for

about 1 hour on a shaker incubator at 16°C for the affinity binding of the His-tagged protein. The beads were separated by filtration and the bound protein was eluted using 10 mM Tris-HCl buffer containing 150 mM sodium chloride and 300 mM imidazole pH 8.0. The elute profile was analyzed by SDS-PAGE. The fractions containing the fused PTPBR7 were pooled and the protein was further purified by size exclusion chromatography using Superdex 75 10/300GL (GE Healthcare). The purity of the protein obtained was determined by SDS-PAGE.

#### N-TERMINAL SEQUENCING

Protein samples containing PTPBR7 were separated on 12% SDS-PAGE and then transferred to Immobilon P membrane at 75 mA for 1 h using transfer buffer that contained 10 mM CAPS, 10% methanol, pH 11. The membrane was washed with water and stained with Coomassie Blue, and the stain excess was removed. The bands corresponding to PTPBR7 were excised from the membrane and sent to N-terminal sequencing (Proteomics facility, University of Leeds).

#### DEGLYCOSYLATION OF PTPBR7 USING PNGASE F

Purified protein samples were denatured in the presence of glycoprotein denaturing buffer at 100°C for 10 minutes, and further incubated with reaction buffer, NP40 and PNGase (NEB) at 37°C for 1 hour. The samples were further analyzed by western blotting using PentaHis monoclonal (against 6XHis tag) antibody.

#### RESULTS AND DISCUSSION

The full extracellular region of PTPBR7 is about 24 kDa and the construct cloned into pHLsec obtained from Dr Hendriks was transfected into HEK293T cells. Large-scale transfection in roller bottles using 0.5 mg of endotoxin free purified plasmid DNA/bottle was used in complex with polyethylenimine (PEI). The conditioned medium was collected 3 days post transfection as the protein is secreted into the medium. The filtered medium was diluted with PBS and incubated with nickel-coated chelating Sepharose on a shaker incubator. The beads with bound His-tagged protein were separated, washed with 10 mM Tris-HCl buffer containing 150 mM sodium chloride, 5 mM imidazole, pH 8.0, and the protein was eluted with 10 mM Tris-HCl buffer containing 150 mM sodium chloride and 300 mM imidazole pH 8.0. The protein was analyzed on 12% SDS-PAGE. A second purification step was performed by size exclusion chromatography using Superdex 75 10/300GL column equilibrated with 10 mM Hepes pH 7.5 buffer containing 150 mM sodium chloride. The elution fractions were analyzed on 12% SDS-

PAGE. The obtained PTPBR7 protein was pure, but presented multiple fragments of lower molecular weight in addition to the full-length protein of 24 kDa (Fig. 1 A). This could be due to the presence of different levels glycosylation of PTPBR7, but the bioinformatics analysis using NetNGlyc server predicted only one N-linked glycosylation site. Deglycosylation using PNGase F, which reduces the heterogeneity in the protein caused by differential glycosylation did not reduce the number of protein fragments (Fig. 1 B) and most of the protein fragments presented lower size than the theoretical MW of ~24 KDa in the absence of glycosylation. This suggested an N-terminal proteolytic cleavage of the secreted protein, ruling out a C-terminal cleavage that would have affected the binding of this C-terminus 6X His-tag fused protein to IMAC matrix. To confirm this, the N-terminal sequencing of several low mass protein fragments was performed. The purified proteins were separated on 12% SDS-PAGE, and electroblotted on to an Immobilon P (Millipore) membrane. The membrane was then stained with Coomassie Blue and the corresponding protein bands were excised from the membrane and analyzed by N-terminal sequencing. The results clearly showed three N-terminal tryptic-like cleavage sites where the protein was cleaved (Fig. 2).

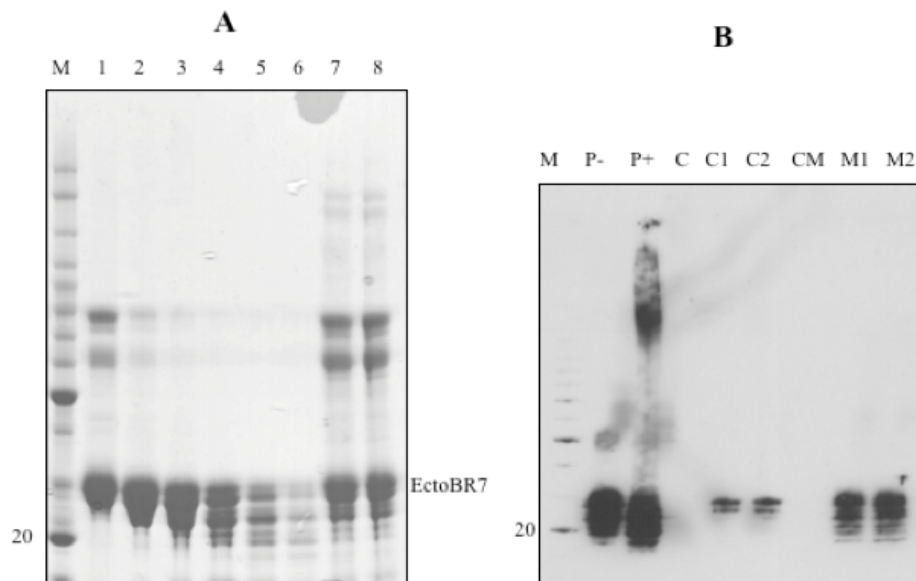


Fig. 1. – Size exclusion chromatography of ecto PTPBR7. (A) SDS-PAGE analysis of peak elution fractions. M: Molecular marker, lanes 1-8 peak elution fractions; (B) Western blot analysis using PentaHis monoclonal antibody. M: molecular marker, P--PNGase F untreated purified protein, P+-PNGase F treated purified protein, C-Control cells (untransfected), C1 and C2- cells transfected with ectoPTPBR7 (2 days post transfection), CM-Control conditioned media (untransfected), CM1 and CM2- conditioned media (2 days post transfection) with ectoPTPBR7.

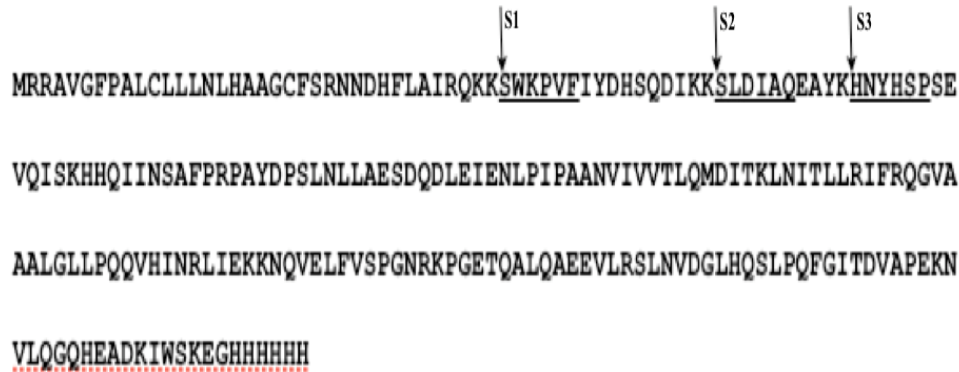


Fig. 2. – Sequence of whole ecto PTPBR7 fused with C-terminal 6X His-tag. Underlined sequences are the result obtained by N-terminal sequencing which is caused by the tryptic-like cleavage at the regions indicated by arrows. S1 (PTPBR7 S1), S2 (PTPBR7 S2) and S3 (PTPBR7 S3) are the short constructs cloned exactly after the proteolytic cleavage sites in order to obtain protein resistant to proteolysis.

In order to obtain a stable extracellular region of PTPBR7, three shorter constructs were obtained, coding for proteins with N-termini corresponding to each of the identified proteolytic sites. The DNA fragments unique restriction sites AgeI and KpnI in the pHLsec vector allowed the insertion of the shorter constructs of ecto region of PTPBR7, named PTPBR7 S1 (SWKPVF), PTPBR7 S2 (SLDIAQ), and PTPBR7 S3 (HNYHSP). PTPBR7 S2 and S3 constructs were used for large-scale protein production. HEK293S cells were used, which allows homogeneous glycosylation of the recombinant protein. The shorter PTPBR7 protein produced by 293S cells was more stable and less prone to proteolytic cleavage (data not shown). Therefore, the expression of PTPBR7 S2 and S3 constructs was performed in 293S cells. The proteins obtained were purified by affinity chromatography (IMAC) followed by a final step of purification by size exclusion chromatography as described earlier. The proteins thus obtained were stable with less proteolytic degradation as compared to the full ecto construct, the shortest construct being the most stable as expected (Fig. 3).

In conclusion, the large-scale expression of the ecto domain of PTPBR7 was obtained in mammalian cells. The full-length extracellular protein of PTPBR7 was unstable during expression and purification processes, due to proteolytic cleavage at multiple sites in the N-terminal region. This was confirmed by protein sequencing of the N-terminal protein fragments obtained after purification. cDNAs coding for N-terminal shorter constructs starting at the proteolytic cleavage sites were cloned, expressed and purified as the full-length ecto BR7. The proteins thus purified were resistant to proteolysis as compared to the full-length ecto region, the shortest construct being the most stable. Consequently, by using this mammalian expression system, we were able to obtain stable pure ecto domain of PTPBR7. This protein can be used for further structural and functional *in vitro* studies.

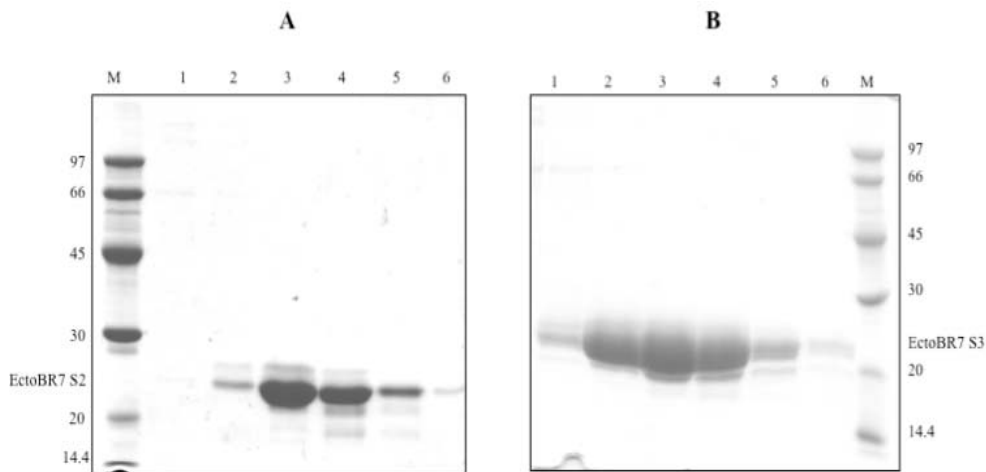


Fig. 3. – Size exclusion chromatography of ecto PTPBR7 S2 and PTPBR7 S3. (A) SDS-PAGE analysis of the peak protein fractions of ecto PTPBR7 S2 construct. M: molecular marker. Lanes 1-6: elution fractions of PTPBR7S2; (B) SDS-PAGE analysis of peak elution fractions of PTPBR7 S3 construct. Lanes 1-6: elution fractions. M: molecular marker.

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