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IDENTIFICATION AND CHARACTERIZATION OF NEW PLASMATIC ISOFORMS OF THE TUMOR SUPPRESSOR GENE PTPRG AND DEVELOPMENT OF NEW SPECIFIC MONOCLONAL ANTIBODIES

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Coordinatore: Prof. Fabio Menestrina

Tutor: Dott. Claudio Sorio

Dottorando: Dott./ssa Elisabetta Moratti

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Abstract

Aims: 1) Identify the presence and characterize the plasmatic isoforms of Receptor Type Protein Tyrosine Phosphatase Gamma (PTPRG) 2) Characterization of new monoclonal antibodies specific for the extracellular domain of the protein.

Background: PTPRG is a broadly expressed enzyme mainly expressed in lung, stomach, esophagus, colon, liver, spleen and kidney. PTPRG maps to chr 3p14.2/21 and has been implicated as a candidate tumor suppressor gene. Loss of eterozigosity, gene deletion, point mutation and promoter hypermethylation has been reported. The majority of the receptor PTP share similar intracellular domains while the extracellular portion exhibits broad structural variation. Rat brain has been reported to express various PTPRG isoforms, including the isolated extracellular region [1]. Previous work from our group suggested the production on an extracellular soluble form of PTPRG in thyroid [2] .

Methods: immunoprecipitation, western blotting, flow cytometry.

Results: We have identified, in human and murine plasma, new PTPRG isoforms, one of which previously characterized by cDNA cloning only in rat [1]. The polypeptides identified have an apparent MW of about 120 kDa (major band) and 90 kDa (minor band) respectively. Surprisingly, also the full length protein, that include a transmembrane region, was identified in the serum and was present in the fraction precipitating at 100000 ×g, suggesting the presence of an exosome-associated form. The major band (120 kDa) was also identified in mouse liver (isolated after saline perfusion in order to remove plasma) and in serum-free conditioned medium derived from HepG2, an hepatocellular carcinoma cell line commonly used for the study of the regulation of hepatic protein synthesis. The 120 kDa isoform was up-regulated by

treatment with ethanol and potassium dichromate, two compounds known to be cytotoxic for liver [3-5]. With the future goal to set up an ELISA-based assay for the rapid screening of soluble PTPRG expression in various physiopathological conditions, we have developed and characterized two new monoclonal antibodies raised against the extracellular domain of PTPRG expressed in eukariotic cells (HEK 293F).

Conclusion: We have described for the first time in human and mouse the presence of soluble, plasmatic forms of PTPRG that appear to be released primarily by liver. The major 120 kDa band appear to be glycosylated and its expression is modulated by stimuli known to produce liver injury.

The production and initial characterization of new monoclonal antibodies capable to specifically recognize this protein here described will permit the set up of new immunoassays and will pave the way to a better characterization of this proteins and its role in health and disease.

Introduction

Kinase/Phosphatase in cell signaling

The importance of phosphorylation and the regulatory role of protein kinases is established. However, it has become apparent that protein phosphatases can no longer be viewed as passive housekeeping enzymes in these processes. Instead, the kinases and phosphatases are partners, and their activities are coordinated in the regulation of signalling responses. The distinct but complementary function of these enzymes is emphasized by recent studies in which the kinases have been implicated in controlling the amplitude of a signalling response, whereas phosphatases are thought to have an important role in controlling the rate and duration of the response [6]. If overexpression of tyrosine kinases or mutations that would render them constitutively active would bring about oncogenicity then, necessarily, overexpression of tyrosine phosphatases would block or reverse transformation, even if the accumulation of experimental evidence have rendered the picture much more complex [7].

Protein Tyrosine Phosphatases

Following the discovery and the purification of the first PTP (PTP1B) in 1988 from human placenta [8] and the identification of the first transmembrane PTP (CD45) [9], many other PTPs have been cloned on the basis of sequence homology in the catalytic domain.

The human genome contains 107 PTPs, with the class I cysteine-based PTPs constituting the largest group. This group can be further subdivided into 61 dual-specificity phosphatases and 38 tyrosine-specific PTP genes, the “classical PTPome”.

Classical PTPs have been further subdivided into receptor (R1–R8) and nontransmembrane (NT1–NT9) subgroups [10, 11]. Twelve receptor PTPs have two catalytic domains (tandem domains), while the remaining PTPs all have a single catalytic phosphatase domain. In tandem-domain receptor protein tyrosine phosphatases (RPTPs), it is the PTP (D1) domain adjacent to the plasma membrane that displays catalytic activity while the PTP (D2) domain is either inactive or has negligible catalytic activity[11]. The functional role of the D2 domain has not yet been defined although possible roles in regulating RPTP stability, specificity, and dimerization have been suggested. Furthermore, all RPTPs, with the exception of PTPRA and PTPRE contain large and diverse extracellular regions that regulate cell contacts and adhesion [12].

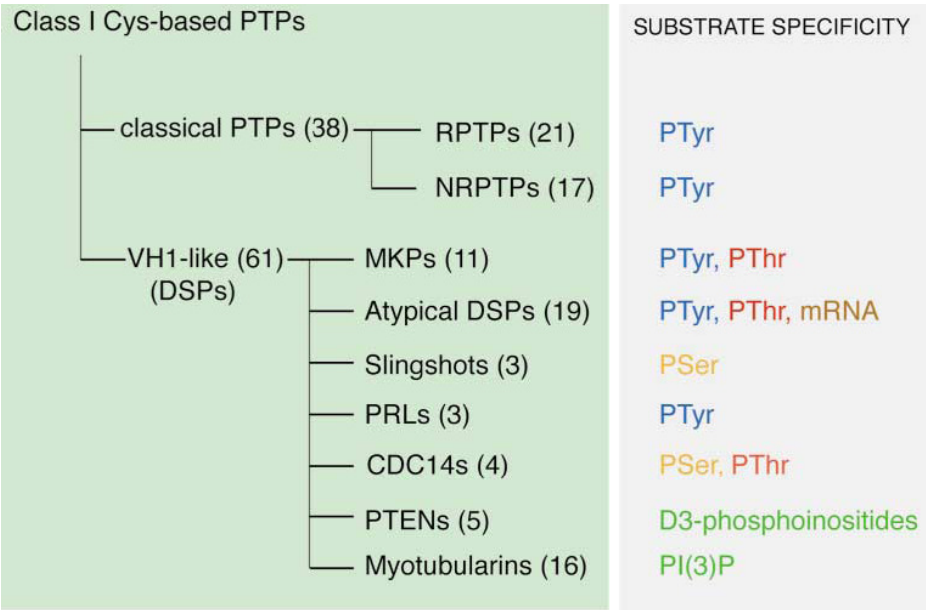


Figure 1: Schematic representation of Cys-based PTPs and their substrate specificity, (part of [10]).

Protein Tyrosine Phosphatase gamma (PTPRG)

PTPRG was originally cloned from human brain stem and placental cDNA libraries using probes derived from the intracellular domain of CD45**.** PTPRG is a gene mapped

in chromosome 3p14-p21 : 61,547,243-62,283,288 in the forward strand. The gene have 11 spliced variants and 6 of this are known to harbor protein coding sequence (http://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000144724).

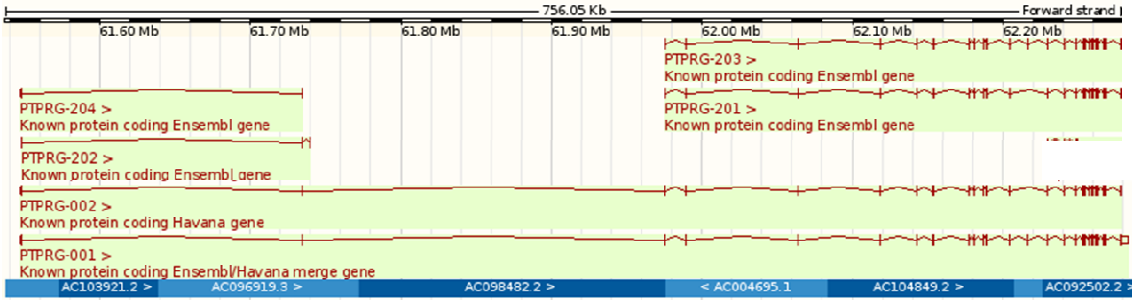


Figure 2: **Schematic representation of PTPRG gene.** Transcripts that are protein coding, from Ensembl database: http://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000144724.

The full length PTPRG is composed of a 30 exons and produce a 1445 amminoacid-length protein. The gene have a 5'UTR region inside the first exon that include the *consensus* sequence for sp1 factor (-410/-405 bp), a CCAAT BOX (-228/-224 bp) and a TATA BOX (-30/-25 bp) upstream the starting codon and have a 3'UTR region at the end of the exon 30.

This PTP belong to a subtype V of the receptor tyrosine phosphatase and possesses an extracellular region, a single transmembrane region and two tandem intracytoplasmic catalytic domains. The extracellular region of this PTP contains a carbonic anhydrase-like (CAH) domain and a fibronectin type three domain which is also found in the extracellular region of PTPRZ.

Expression of the PTPRG protein in cells and normal and neoplastic tissues

Membranous and⁄or cytoplasmic PTPRG expression was detected in leukocytes, including hemopoietic precursors, the vast majority of epithelial cell types and

endocrine cells of various organs [13] [14] [15]. In summary, in humans PTPRG was described as a marker for $CD34^+$ cells, monocytes, dendritic cells and specialized macrophages. It is also expressed in all the epithelia of the gastrointestinal tract (including stomach, small and large bowel mucosa and appendix) and most cells of the genitourinary system (proximal and distal tubules of the kidney, endometrium, fallopian tubes, prostate glands). PTPRG expression was also found in breast and ducts of parotid and sweat glands. In the liver, hepatocytes were strongly immunopositive, whereas bile ductules were faintly stained. Squamous epithelia, including epidermis, oral cavity, oesophagus and uterine cervix, strongly expressed PTPRG. Epithelial cells showing inconsistent and rather faint immunoreactivity included urothelium, bronchial mucosa, gallbladder and large bile ducts. In the lung, the epithelia of bronchioli, pneumocytes and macrophages were all immunoreactive. Neither T- or B-lymphoid cells appear to express PTPRG as assessed by immunohistochemistry of thymus and lymph nodes, while a strong signal was associated with sinus macrophages. Remarkably high expression of PTPRG was found in endocrine cells of the gastrointestinal tract, pancreatic islet of Langerhans, adrenal medulla and thyroid. Interestingly, thyroid follicular cells were stained with both intracellular and extracellular antibodies, while the colloid was strongly positive only using the extracellular one. Among non-epithelial cells, PTPRG was expressed by endothelia of various organs, including spleen sinusoids and high endothelial venules in lymph nodes. Immunoreactivity was also intense in the smooth muscle layer of the wall of larger vessels. Variable staining was detected in fibroblasts of connective tissue. Striated and smooth muscle cells were strongly immunopositive for PTPRG, while fat cells were immunopositive, but inconsistently so. In the peripheral nervous system, PTPRG was regularly expressed by ganglion cells within the myenteric plexus. In the central nervous system, positivity was detected in some scattered cells with elongated

nuclei and a few dendritic cell processes that probably represent elements of the microglia. Purkinje cells of the cerebellum and some neuronal elements were also positive.

PTPRG in neoplasia

PTPRG is a candidate tumor suppressor gene and mutations, deletion or loss of eterozigosity are associated with different type of tumor.

In **lung carcinoma** (LC) tumor samples PTPRG expression was down-regulated in tumor cells only, but still expressed in the normal, interstitial cells. The PTPRG suppression in 50% of the human lung adenocarcinomas confirms and extends on the protein level the suggestion of LaForgia et al [16] that reported PTPRG gene deletion in two out of five human lung adenocarcinoma cell lines. Such non-sporadic deletions of PTPRG are thought to signify the presence of a gene(s) whose functional inactivation is involved in the malignant process.

In **ovarian cancer** the expression of PTPRG was reduced in both the tumor cells and the normal interstitial cells [2, 15].

The translocation t(3;8) of the **familiar renal cell carcinoma** (RCC) occurs inside the 5' end of PTPRG gene but the 6-Kbp coding sequence was not disrupted by the translocation. Since tumor suppressor genes involved in specific type of familial cancer are often involved in development of sporadic tumors of the same type, it seemed likely that if the 3p14 locus encompassing the translocation breakpoint harbors a suppressor gene, this region would also be involved in allele loss in sporadic RCCs. 89% of RCCs exhibit a common region allele loss[17].

Weak PTPRG expression in **breast cancer** compared to normal tissue correlated with a higher estrogen receptor status ERα and a lower ERβ expression. Inhibition of PTPRG

RNA expression by estradiol in primary-cultured normal breast cells and in breast cancer cell lines suggesting that PTPRG may be a potential estrogen-regulated tumor suppressor gene in human breast cancer, which might thus play an important role in mammary epithelium neoplastic transformation [18]. In keeping with this finding, over-expressing PTPRG can antagonize the estrogenic effects of 17β-estradiol on cell proliferation which indicates its anti-estrogenic effects on human breast cancer[19].

Mutational analysis of the tyrosine phosphatome in **colorectal cancer** display 83 somatic mutation in six PTPs including PTPRG where 8 point mutation (7 missense and 1 LOH) were detected [20].

Malignant T cells of patients with **CTCL** display widespread promoter hypermethylation associated with inactivation of several tumor suppressor genes involved in DNA repair, cell cycle and apoptosis signaling pathways including PTPRG[21].

The PTP expression profile obtained in **gastric cancer** sample revealed a strong representation of a few PTPs such as PTPRG, PTPRA and PTPRC, which occupied more than 70% of the clones that were analyzed. PTPRG expression level was high in normal human gastric tissue while is reduced in cancer[22]. Difference exists in DNA methylation between primary tumor and metastatic lymph nodes of gastric cancer, where the gene seems to be more methylated in the metastasis than in the primary tumor that suppress the expression of the gene[23].

PTPRG was identified as a candidate tumor suppressor gene in **nasopharyngeal carcinoma** (NPC) by differential gene profiling of tumorigenic and nontumorigenic NPC. Down-regulation of this gene was found in NPC cell lines and tumor biopsies. Promoter hypermethylation and loss of heterozygosity were found to be important

mechanisms contributing to PTPRG silencing. PTPRG overexpression in NPC cell lines induces growth suppression and reduced anchorage-independent growth in vitro [24].

Type of tumor	*Type of mutation*
Familial renal cell carcinoma (RCC) t(3;8)	Translocation, LOH
Lung Cancer	Down regulation by gene deletion, LOH or protein down modulation
Breast cancer	Down regulation
Colorectal Cancer	Point mutations
CTCL (cutaneous T-cell lymphoma)	Promoter hypermethylation
Gastric cancer	Down expression of the protein
Nasopharyngeal carcinoma	Promoter hypermethylation, LOH

Table 1: **Summary table of the mutations reported for each type of tumor.**

Regulation of extracellular domain of Receptor Type PTP (PTPR)

The ectodomains of some PTPRs are cleaved off by specific protease and remain bound extracellularly to the citoplasmic region of these PTPs [25]. In addition, these ectodomains may be shed, and thus may have a function by themselves, acting as ligands, independently of the catalytic activity of the enzyme. The most appealing function of the ectodomains that may bind ligands that somehow induce an alteration of the intracellular PTP activity.

PTPRF (LAR) is a 1881 amminoacid protein with a MW of about 200 kDa that is cleaved intracellularly into two subunits: P-subunit with a intracellular portion,

transmembrane peptide and a short segment of the extracellular region that are then non-covalently associated to the E-subunit (consisting of the extracellular domain of the protein). The extracellular portion can be released from the cell surface [25].

PTPRA and PTPRE are two other PTPs subjected to proteolytic cleavage. The cleavage is calpain-mediated and produce, from the larger PTPRA and PTPRE proteins, a processed form of about 66kD and 65kDa, respectively. The cleavage occurs in close proximity to the wedge domain, a region that participates in significant intermolecular interactions in the course of phosphatase dimerization. Proteolytic cleavage and subcellular re-distribution of these protein can affect the physiological function with a gain or loss of function. The truncated form of PTPRA is less able to dephosphorylate a substrate than the full length PTPRA, but the translocation of the truncated protein can access to potentially new substrate in the cytosol[26].

PTPRU is a phosphatase that undergoes posttraslational cleavage into two non-covalently associated subunits [27]. The concentration of the full length protein is up regulated in cell density-dependent inhibition of growth and the increase of the expression is associated with an increase in its proteolytic cleavage. This processing of PTPRU may have an important role in contact inhibition of cell [28].

After the cloning of PTPRK, a tyrosine phosphatase of about 210 kDa, in 1993, the generation of a product of an unexpected smaller size was observed. PTPRK protein consists of two subunits that are noncovalently attached to each other: the transmembrane P subunit (100 kDa) that harbors two PTP domains and the extracellular E subunit (120 kDa) that covers most of the extracellular sequence. Both subunits are generated from one precursor protein by proteolytic processing at the dibasic cleavage site (RTKR) located within the membrane proximal fibronectin type III domain.

The two cleavage products remain associated after cleavage and may be considered subunits of a single complex; this association is not mediated by a disulfide linkage [29]. The processing of the protein is accomplished by sequential proteolytic processing by three proteases at three sites. While the first cleavage by furin participates in RPTP maturation in the secretory pathway, cleavage at the second site by ADAM 10 (nearest to the transmembrane in the extracellular domain) results in the shedding of the phosphatase extracellular fragment. Finally, intramembrane proteolysis at third site by γ-secretase releases the intracellular domain of the protein to the cytoplasm. In this proteolytic pathway, processing by ADAMs may play a major regulatory role [30].

It is known that three isoforms of Ptprz are generated by alternative splicing from a single *Ptprz* gene in rat: two transmembrane isoforms, Ptprz-A (full length isoform) and Ptprz-B (a deletion variant missing an 860 amino acid in the extracellular domain juxtamembrane), and a secretory isoform named Ptprz-S[31].

Ptprz-A and Ptprz-B can undergo further processing by metalloproteinase-mediated ectodomain shedding, which releases the extracellular fragment $Z_{A/B}$-ECF(extracellular fragment), from the cell surface and produces the membrane-tethered counterpart ZΔE. ZΔE is digested by PS/γ-secretase, and the cytoplasmic fragment Z-ICF (intracellular fragment) is released from the plasma membrane and is detected not only in the cytoplasm but also in the nucleus, suggesting a novel signaling pathway driven by Ptprz [32].

Finally four different isoforms of Ptprg have been identified in rodents of which one is represented by a soluble form.

Ptprg isoforms are derived from alternative splicing, and are designated as ptprg-A (full length isoform), ptprg-B (lacking the intracellular juxtamembrane 29 amino acid) [33],

ptprg-C that have only one phosphatase domain and ptpr-S an extracellular variant of the protein that is secreted into culture medium when expressed in COS7 cells [1].

Aims of the study

In order to better study the role of plasmatic PTPRG isoforms we needed to determine the type of protein present, its origin and to develop suitable tools for its identification and measure, ideally suited for high-throughput assays. To this end we also needed to generate new monoclonal antibodies. In this thesis I will go through a series of data that will try to answer to these critical issues.

Material and methods

Patient samples:

Preparation of plasma and serum

Plasma was obtained from healthy blood donors from buffy coats or from whole peripheral blood. Peripheral blood was drawn from normal healthy donors into EDTA tube and spun down at first at 335 ×g for 15 minutes and then the supernatant at 3000 x g for 15 min in a Heraeus Megafuge 1.0R. Plasma was drawn off and used immediately or frozen at 80 °C until use.

The serum was processed as the plasma sample after coagulation of blood sample in EDTA free tube.

All the samples derived from the transfusion blood bank of the University and Hospital Concern of Verona (Italy).

Antibodies

The antibodies used were:

1) anti-PTPRG rabbit polyclonal antibody P4 (Rb anti-P4) obtained against immunity of rabbit with a synthetic peptide (CGSDPKRPEMPSKKPMSRGDRFSED amino acids 687-710 named P4) coupled, through an NH2-terminal cysteine, to KLH (Sigma Chemical Co., St. Louis, MO). [33].
2) anti-PTPRG chicken polyclonal antibody (Ch-PTPRG) obtained against the synthetic peptide (CZ NED EKE KTF TKD SDK DLK (amino acids 390-407) and produced by Aves Labs (Tigard, OR, USA) [34].

3) anti-human/mouse/rat PTPR gamma monoclonal antibody, MAB4608 (R&D Systems, Minneapolis, USA).
4) polyclonal anti-PTP gamma rabbit antibody (ab37525) a KLH-conjugated synthetic peptide (10-30 amino acids in length) in the region of 791-807 of Human PTP gamma (Abcam Inc., Cambridge, USA).
5) two monoclonal antibodies anti PTPRG extracellular domain developed in our laboratory (described in following paragraph).
6) rabbit isotype control IgG: Chrompure Rabbit IgG, whole molecule (011-000-003 Jackson Immuno Reserch laboratories, inc., Baltimore Pike , USA).
7) chicken isotype control (IgY, chicken IgG) from pre-immunized chicken from Aves Labs (Tigard, OR, USA).
8) mouse IgG (015-000-003 Jackson Immuno Reserch laboratories, inc., Baltimore Pike , USA) and mouse IgM isotype control (Sigma St. Louis, MO, USA).

All the antibodies were used according to the manufacturer's instructions.

Antibody	Class	Epitope	Source	Tested application
Chicken PTPRG	IgY	390-408	Sorio, C. *et al.* (1995)*	IP, WB, IHC, Flow
Rabbit PTPRG- P4	IgG	687-710	Mafficini, A. *et al.* *(2007)***	IP, WB, IHC, Flow
Rabbit ab37525	IgG	791-807	Abcam (Cambridge,UK)	WB, ELISA
Mouse Mab4608	IgG_{2B}	111-541	R&D Systems, (Minneapolis, USA)	WB
Mouse Mab 6E2-B6	IgG_{1B}	ECD	This theses	IP, Flow
Mouse Mab12G5-B10	IgM	ECD	This theses	WB, Flow

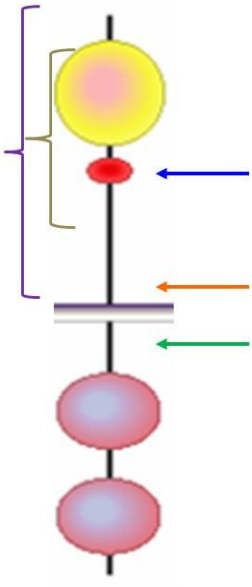


Table 2: Schematic representation of antibody against PTPRG utilized in this study. Epitope: aminoacid number of epitope, based on **ENST00000474889 protein sequence.** Tested application: IP= immuno-precipitation, WB= western blot, IHC= immunohistochemistry, Flow=

flow cytometry. The PTPRG schematic image (right) display where the epitopes of the antibody are localized within the predicted protein sequence The colored arrows localize the epitopes relative to the antibodies shown in the table with the corresponding colors [*33, **34].

Recombinant Protein and monoclonal antibodies

The extracellular domain of PTPRG linked to mouse Fc (PTPRG-ECD-Fc), fusion protein between PTPRG extracellular domain (amino acid 1-736) and mouse-Fc IgG_3 (aminoacid 1-237) used as protein tag, and the control protein Fc, were produced in our laboratory in HEK 293F cell line and affinity purified following standardized protocols. The recombinant protein PTPRG-ECD-Fc was used as epitope for the immunization, as immunogen for the ELISA screening and as standard for the glycosylation experiments. The mouse hybridoma cell lines were produced by ARETA International srl (Gerenzano VA, Italy) from BALB-C mouse that has been challenged with PTPRG-ECD-Fc. The screening of the different clone and subcone produced by ARETA were first evaluated by direct ELISA using the purified protein as antigen and the control protein Fc in order to remove all the clone reacting against the tag. Subclones were screened on the basis of capability to recognize the native antigen by flow cytometry. Two clones: Mab 6E2-B6 (IgG) and Mab 12G5-B10 (IgM) were chosen for further analysis.

Cell culture

HepG2 cell (human, Caucasian, liver, carcinoma, hepatocellular, ATCC HB 8065) were cultured in EMEM containing 2mM glutamine, 1mM Na pyruvate and 10% heat-inactivated Fetal Calf Serum (FCS) or cultured in serum free medium. HepG2 cell were cultured in SF medium with 25, 6.25 µM of potassium dichromate (Sigma-Aldrich, Milan, Italy) and 400, 200, 100 mM ethanol (VWR, Milan, Italy).

Transfected HEK 293F cells were cultured in RPMI 1640 containing 10% heat-inactivated Fetal Calf Serum (FCS), 4mM glutamine, and 0,5 mg/ml of hygromycin (Invitrogen Milan, Italy) as selective agent or cultured in serum free medium CD293® (Gibco, Milan, Italy) with 50 µM di β-mercaptoethanol and 0,5 mg/ml of hygromycin (Invitrogen, Milan, Italy). All the cultured cells were grown at 37°C, in 5% CO_2.

Cells and tissue lysate

$20x10^6$ cells/1 ml were solubilized in lysis buffer (LB) containing 50 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 200 µM $NaVO_4$, 1 mM EDTA, 1 mM DTT, 1 mM NaF, Complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche) incubated for 20 min. in a rotating wheel at 4°C and centrifuged for 30 min at 4 °C at 13000 ×g in a Biofuge Pico, (DJB Labcare Ltd. UK). Normal tissues isolated from PBS-perfused mice were frozen in liquid nitrogen vapors and stored at –80°C. A series of 20 µm cryostat coronal sections were lysed in cold LB and processed as previously described. All the procedures were carried out at 4°C.

Ultracentrifugation and supernatant precipitation

Serum free conditional medium (SFCM) and plasma were ultra-centrifuged at 100000 ×g for 2 h at 4°C in analytical ultracentrifuge Beckman Optima XL-A (Beckman counter Inc., Brea, CA, USA). The supernatant was transferred in a fresh tube while the pellet was resuspended in SDS-PAGE sample buffer (SB). Proteins were precipitated from the SFCM by addition of trichloroacetic acid (TCA) to a final concentration of 10% under continous stirring at 4°C overnight. Following centrifugation at 3000 ×g for 30 minutes at 4° C, the pellet was washed twice with a mixture of cold acetone / methanol 8:1 and pelleted at 13000 ×g for 15 minutes at 4°C. The pellet was dried in a

speed-vac (Savant SVC 100H Speed Vac Concentrator, Phoenix Equipment Inc. AZ, USA) for 30 minutes. The dried pellet was the resuspended in 20 µl of SB. Plasma samples was ultra-centrifuged as SFCM and treated as indicated.

Flow cytometry

Cells (monocyte and Hep G2) were washed twice with PBS buffer, blocked with 1 µg of Human IgG (Sigma St. Louis, MO, USA) for 15 minutes at RT and then incubated with Ch-PTPRG, Rb anti-P4 antibody (2 µg/10^6 cells) or mouse IgG 6E2-B6 / IgM 12G5-B10 (1 µg/10^6 cells) and with suitable isotype control for 1 hour on ice. After one wash with PBS buffer cells were stained with goat anti-chicken Alexa488, goat anti-rabbit Alexa488 or goat anti-mouse Alexa488 IgG/IgM (Invitrogen, Milan, Italy) for 30 min at RT. Flow cytometry was performed on a Becton Dickinson FACScan®. Analysis of data was performed with FCS Express V3 software (De Novo Software).

Purified cells (monocyte) were purified by Percoll (Pharmacia Uppsala, Sweden) gradient centrifugation from leukocyte rich buffy coats obtained from human blood of healthy donors, as described elsewhere [35].

Immunoprecipitation with Cyanogen-bromide activated Sepharose 4B

4 mg antibody /0,286 g gel and the same quantity for an isotype control was coupled to Cyanogen-bromide activated Sepharose 4B (Sigma, Missuri, USA) according to the manufacturer's protocol.

Plasma samples from blood donors and patients were diluted 1:5, 1:10, 1:20, 1:40 in PBS/TX100 1% to a final volume of 500 µl and added with 5 µl of Cyanogen-bromide activated Shepharose 4B with Rb anti-P4 and its control (RbIgG isotype). After three hour in a rotating wheel, beads were washed three time with PBS and the pellet were

mixed with SB (to a final concentration of 40 mM Tris-HCl pH 6.8, 183 mM β-mercaptoethanol, 1% SDS, 5% glycerol) and denatured at 95°C for 10 minutes.

Immunoprecipitation with Protein G Sepharose 4 Fast Flow

Total protein content was assessed using Bradford assay [36] (Sigma, Missuri, USA). Serum-free conditioned medium (SFCM) or cell lysate (250 µg of total protein for each sample) were incubated with 3 µg of specific antibodies for 3h at 4°C. Beads (cat n:17-0618-01 GE Helthcare, Little Chalfont, UK) were centrifuged and washed one time in Tris-buffered saline (TBS) and three times in LB or serum free medium before the immunoprecipitation. 20 µl of protein G-Sepharose (Sigma St. Louis, MO, USA) for each sample was added and left for 1 h at 4°C with gentle rocking. Following three washes in TBS buffer the pellet was resuspended in SB and incubated at 95°C for 10 minutes and subjected to SDS-PAGE.

Deglycosylation

Deglycosylation of purified recombinant extracellular domain of PTPRG linked to Fc (ECD-PTPRG-Fc) and the immunoprecipitated plasmatic PTPRG was carried out with the use of PNGase F (Boehringer, Mannheim, Germany) according to the manufacturer's protocol.

Western blot

Cell lysate, serum free supernatant of cells (HEK 293F transfected with a PTPRG-ECD-Fc and Fc carrying vectors) and tissues lysate were resolved on 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane were blocked with 3% BSA for polyclonal antibody and 5% BSA

for the monoclonal, in TBS, 0.05 % Tween-20 (TBST) and probed with suitable antibodies at 1 µg/ml in 1% BSA TBST buffer o.n. After washes in TBST, the membrane was then incubated with the following secondary antibody: 1) donkey anti-rabbit IgG-HRP (GE Helthcare, Little Chalfont, UK), 2) goat anti-mouse-HRP conjugated (GE Helthcare, Little Chalfont, UK) or 3) with anti mouse Fc-HRP conjugated (NOVUS biological, Littleton, CO, USA) for 1 h. After washing with TBST, the signal was detected with the enhanced chemiluminescence kit (Millipore Corp., Bedford, MA).

Mice perfusion and tissue collection.

Male C57BL/6J wild-type mice, 5–8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained under specific pathogen-free conditions. All experiments were performed in accordance with the Animal Care and Use Committee guidelines.

The mice were anesthetized with an i.p. injection of Ketamine/Xilazina (80-100mg/kg Ketamina and 10mg/kg Xilazina in PBS). Following abdominal incisions, the heart of the mice were perfused with Phosphate Buffer Saline (PBS), 22 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ through the left ventricle of the heart using Masterflex Pump Controller (Cole Parmer Instrument Co., Chicago, IL, USA) at a flow rate of 4 ml per minute. This flow rate approximates the murine physiological blood pressure. When all the blood was replaced with the saline solution organs (brain, thymus, heart, lung, kidney, spleen, liver and pancreas) were removed, snap-frozen with dry ice, and stored at -80°C. A series of 20 µm cryostat coronal sections were lysed in LB. Quality of the tissue was checked with Hematoxyilin-Eosine staining. Equal protein amounts were mixed with SB, subjected to SDS-PAGE and electroblotted on nitrocellulose

membrane. Immunoblotting was done with two polyclonal antibodies (Rb anti-P4 and Ch-PTPRG).

MTT assay

Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (Sigma, Missuri, USA) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at 570 nm by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which have been dissolved in DMSO and the absorbance read at 570 nm.

HepG2 cells were seeded at 10.000 cell/well in 96-well flat bottom culture plates and incubated overnight, the day after were exposed to different concentration of potassium dichromate and ethanol diluted in complete medium and serum free medium and incubated for 24 h. The SFCM were collected and processed . About 10 µl of MTT (5 mg/ml) was added to each well and incubated for 3 h. The formazan dye formed was extracted with 100 µl of DMSO.

Results

Detection of PTPRG isoform in plasma/serum

We performed immunoprecipitation (IP) of six different mouse serum and a lot of human plasma samples (**fig 3 B** display one representative sample) with polyclonal anti-PTPRG-RbgG P4 linked to Cyanogen-bromide activated Sepharose 4B and subsequent detection by Immunoblotting with the same antibody that readily displayed the full length protein**,** a specific band about 120 kDa and a band of 90 kDa (**Fig 3**).

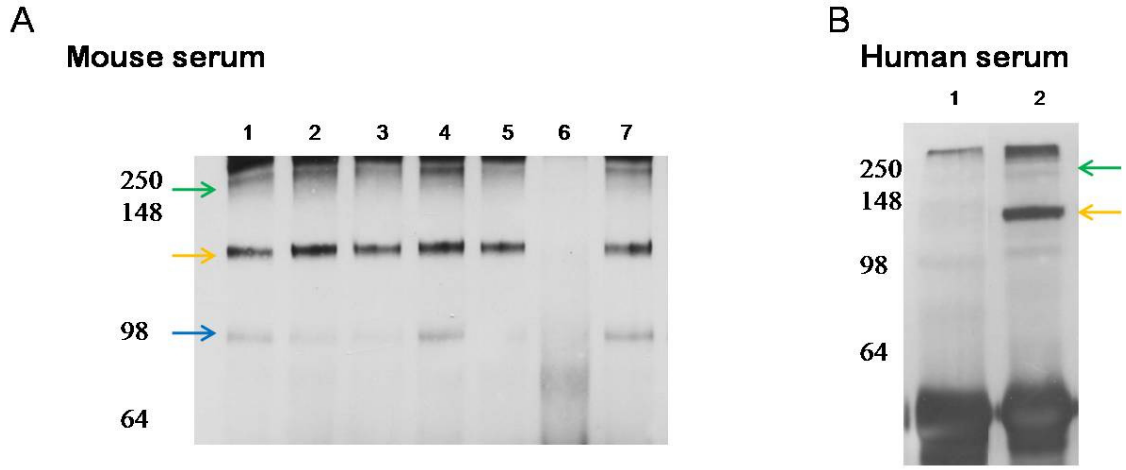


Figure 3: Plasmatic isoforms. A: Immunoblotting with Rb anti-P4 of immunoprecipitated serum samples diluted 1:20 in PBS/Tx_{100} 1%. lane 1-5 ,7: IP with Rb anti-P4. lane 6: RbIgG isotype control. **B**: Immunoblotting with Rb anti-P4 of immunoprecipitated human serum diluted 1:20 in PBS/Tx_{100} 1%. Lane 1 IP with isotype control lane, 2: Rb anti-P4. Green arrow: full lenght protein, yellow arrow: 120 kDa isoform, blu arrow: 90 kDa isoform.

For a better understanding of PTPRG processing we first evaluated if the full length of the protein found in plasma was linked to the plasma membrane in the form of small particles released by the cells and known as exosomes.

To this purpose we have analyzed plasma samples before and after ultra-centrifugation at 100000 ×g, a speed that is known to preciptate these nanoparticles known to be present in plasma (**fig 4**). As we could detect the FL protein in the pelleted fraction, we can conclude that the FL protein is likely associated to plasma-membrane particles defined as "ectosome" or "exosome" [37]. Both 120 kDa and 90 kDa isoforms are present in plasma after ultra-centrifugation, and therefore represent soluble plasmatic isoforms.

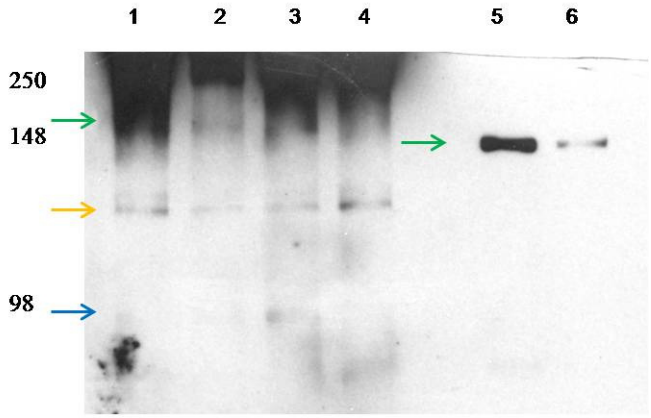


Figure 4: **Identification of a plasmatic PTPRG isoform**. Immunoblotting with Rb anti-P4 antibody of immunoprecipitated serum samples (serum sample 1: lane 1,3,5; serum sample 2: 2,4,6) diluted 1:10 in PBS/Tx_{100} 1% with the same antibody. Lane 1,2: serum sample before 100000 ×g ultracentrifugation. Lane 3,4: same plasma sample after ultracentrifugation. Lane 5,6: pellet derived from the ultracentrifugation.

PTPRG glycosylation

Based on sequence analysis we predicted an extracellular domain form of 81 kDa while the protein we detected has an apparent MW of about 120 kDa. This discrepancy could be explained by the occurrence of glycosylation, a typical feature in extracellular domains of many receptor-like polypeptides. Indeed the treatment with Glycopeptidase F (PNGase F), a deglycosilant enzyme, determined a reduction of MW from 120 to about 100 kDa. PGNaseF cleaves asparagines linked high mannose as well as hybrid

and complex oligosaccharides from glycoproteins. It deaminates the asparagine to aspartic acid, but leaves the oligosaccharide intact. The same shift in MW was observed using the PTPRG-ECD-Fc (**Fig 5).** The slightly higher molecular weight of the recombinant protein is due to the presence of a fusion cDNA with a murine Fc tag.

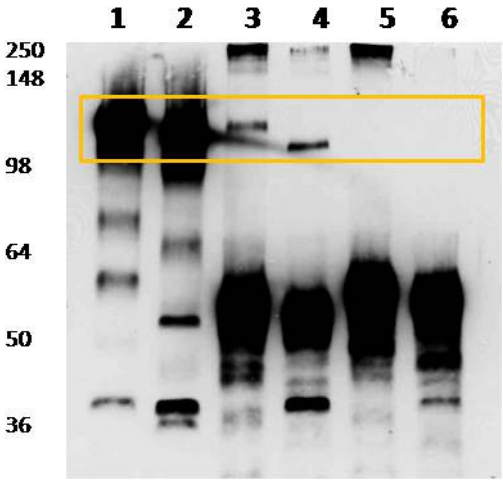


Figure 5: **Extracellular PTPRG is present as a glycoprotein in human plasma.** Immunoblotting with Rb anti-P4 of purified recombinant PTPRG-ECD-Fc lanes 1,2 and immunoprecipitated plasma samples (lane 3-6) and. Lane 1-4: IP Rb anti- P4, lane 5-6: IP RbIgG isotype controls. Lanes 2, 4, 6: same samples of lanes 1, 3 and 5 treated with the deglycosilating enzyme PNGase F.

PTPRG isoforms

For a better characterization of the soluble isoforms of PTPRG we performed immunoprecipitation of plasma followed by immunoblotting using four different antibodies (whose features are described in **Tab 2**). **Figure 6A** summarizes the results: all the antibodies recognize the full length protein (green arrow), the 120 kDa protein is recognized by all the antibodies raised against the extracellular domain (lane 1,2 A and B). The 90 kDa isoform (light blue arrow) is detected with all the antibody recognizing PTPRG including the intracellular epitope, suggesting the conservation of the transmembrane domain in this isoform (lane 3). The Abcam antibody (the more N-

terminal antibody) recognize also a band around 80 kDa that need to be better characterized. In **fig 6B** a IP from plasma followed by a imunoblotting with the same antibody (Ch-PTPRG) is shown. This experiment permit to recognize a doublet around 120 kDa probably due at different state of glycosylation of the ECD, known to be highly glycosylated. One of these bands is apparently not recognized by the anti-P4 antibody.

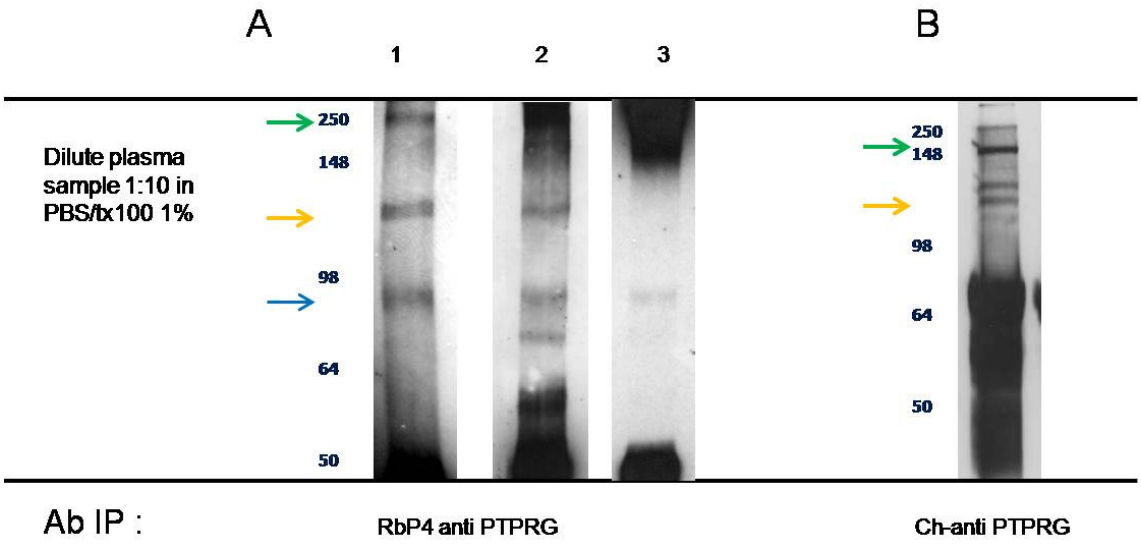


Figure 6: **Characterization of plasmatic PTPRG isoform**. **A**:Immunoblotting with: Rb anti-P4 (lane 1), mouse monoclonal R&D (lane 2), rabbit polyclonal Abcam (lane 3) of immunoprecipitated human plasma samples. Lanes 1-3: IP with Rb anti-P4 anti PTPRG. **B**: Immunoblotting with chicken polyclonal anti PTPRG of the plasma sample immunoprecipitate with the same antibody. Green arrow: full lenght PTPRG, yellow arrow: PTPRG 120 kDa isoform, light blue arrow: 90 kDa isoform.

Expression in murine tissues

The second aim of this study was the identification of the tissue/s that produce the 120 kDa isoform. As a plasmatic form of the protein has been detected, we needed to remove any trace of plasma from tissues before performing the analysis. This was accomplished by perfusion of all the organs with saline as described in material and methods section. The full length protein is detectable in almost all the tissues examined.

A fainter band was detected in pancreas while no protein was detected in kidney. A 120 kDa protein was readily detectable only in liver (**fig 7**).

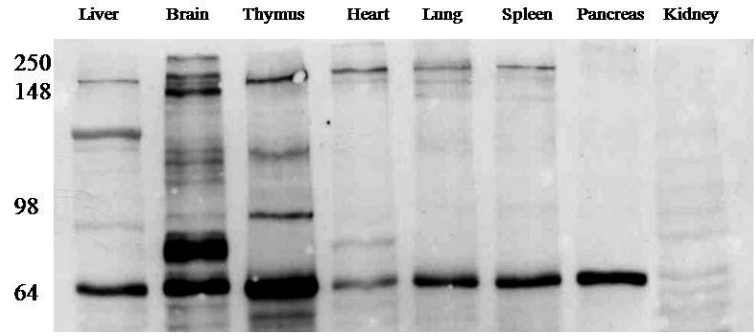


Figure 7 PTPRG expression in mouse tissues. Immunoblotting with Rb anti-P4 antibody. 20µg of lysate from perfused tissues were loaded on 10% SDS-PAGE.

In order to better characterize the antigen, immunoprecipitation followed by western blotting was also performed on the same samples (**fig 8**).

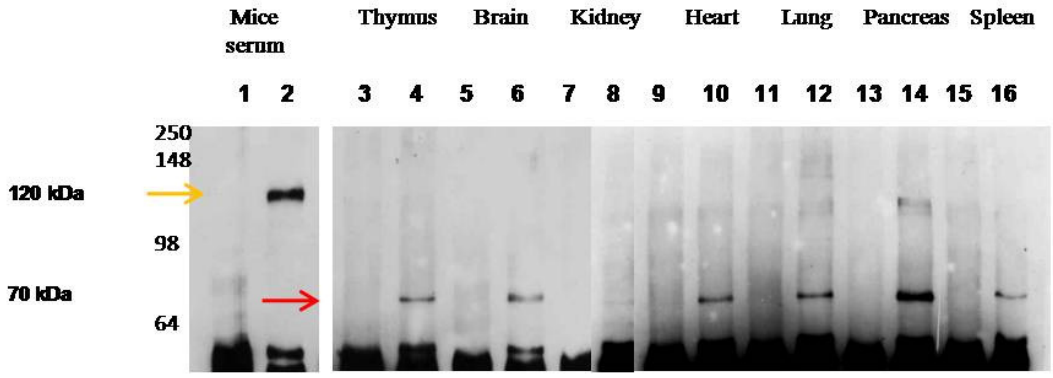


Figure 8: **Characterization of PTPRG isoforms in mouse serum and selected organs**. Immunoblotting from IP mice serum and mice tissue lysate (250 ug each for the tissues). Lane 1,2 :mice serum 1:IP CN-Br RbIgG isotype , 2: IP CN-Br Rb anti-P4. Lanes 3, 5, 7, 9, 11, 13, 15: IP Protein G Sepharose Rabbit IgG Isotype control. Lane 4, 6, 8, 10, 12, 14, 16: IP Protein G Sepharose Rb anti-P4. Western blotting was reacted with Rb anti-P4 antibody.

None of the tissues investigated express detectable levels of the 120 kDa isoform. In all the tissue examined a single band corresponding to the full-length membrane form of

PTPRG was detected. Moreover, immunoblot assay highlight a band of approximately 70 kDa in all the tissues investigated (with the exception of kidney) that need to be better characterized.

A more detailed analysis of liver samples highlight the presence of two specific bands of approximately 120 and 90 kDa that are also detected in plasma/serum (**Fig 9A**). The 90 kDa band react with and antibody raised agains a juxtamembrane intracellular domain, thus indicating that this isoform retains epitopes located on both sides of the transmembrane region (**fig 9B).**

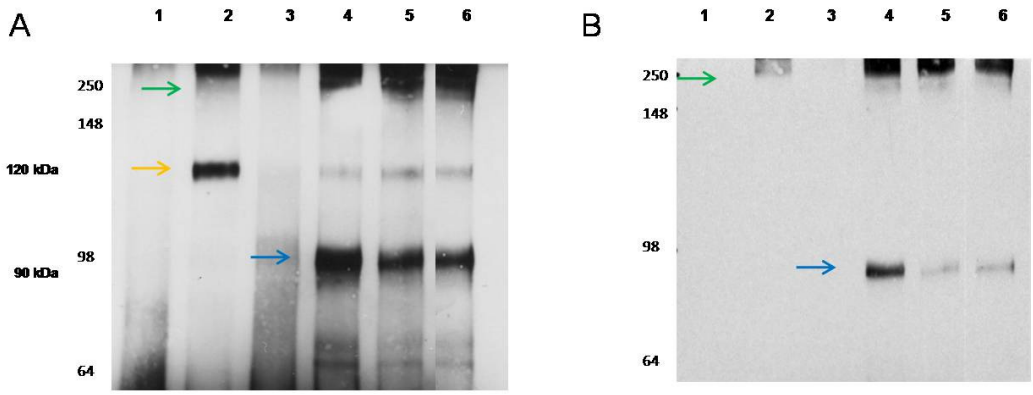


Figure 9. **PTPRG isoforms present in liver.** Panels A and B : Immunoprecipitation from mouse serum (lane 1,2) and liver lysates (3-6) IP with Rb anti-P4 (lane 2, 4-6) or with RbIgG isotype (lane 1,3). Lane 1,2 :IP CN-Br , 3-6 :IP Protein G Sepharose.
Panel A: WB with Rb anti-P4 reacting against an epitope located in the extracellualr domain. Panel B: WB with polyclonal anti PTPRG (791-807 R&D) reacting against an epitope located in the intracellular domain of PTPRG. Yellow arrow :120 kDa and light blu arrow: 90 kDa isoform.

Hep G2 cell model

Searching for experimental models capable to overcome the typical limitations of primary tissues we have chosen the HepG2, a cell line derived from an hepatocellular

carcinoma. HepG2 cells are used routinely for a variety of biochemical and cell biological assays addressing hepatocyte functions. These cells secrete a variety of major plasma proteins; e.g., albumin, transferrin and the acute phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin and plasminogen and represent the most commonly used cell line for examining the regulation of hepatic protein synthesis. We have first evaluated the protein expression by flow cytometry (**fig. 10**).

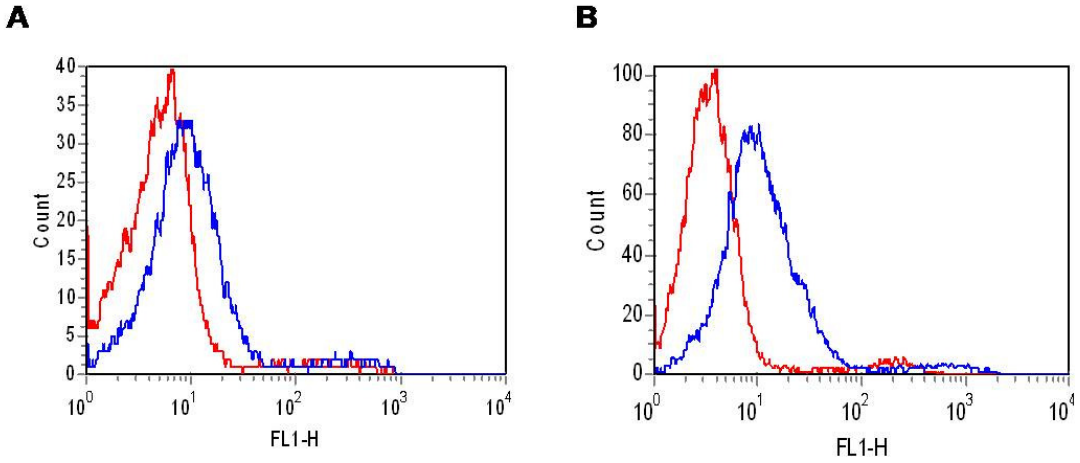


Figure 10: PTPRG expression in Hep G2. Flow cytometric analysis of Hep G2 cell line. Staining with **A**: Rb anti-P4 **B**: Ch-PTPRG (blue lines). Isotype control staining is shown in red.

Subsequently cell lysate and conditioned medium (protein precipitation from serum free (SF) medium of HepG2) were analyzed. The result confirmed the presence of 120 and 90 kDa found in plasma and liver tissue samples with the excepition of the full length protein in SFCM (**fig. 11**).

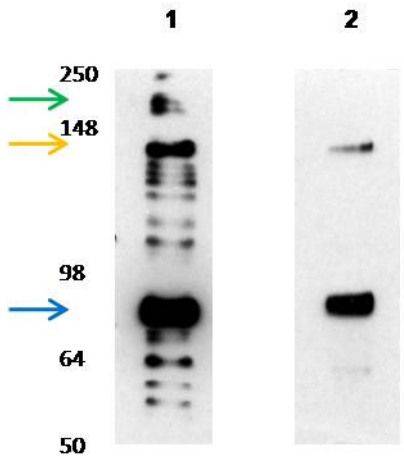


Figure 11: **Expression of PTPRG isoforms in Hep G2 cells**. Lane 1: 10 µg of Hep G2 cell line total cell lysate. Lane 2: serum free supernatant of HepG2 after 100000 ×g ultra-centrifugation and TCA precipitation. WB with Rb anti-P4. Green arrow: FL protein, yellow arrow : 120 kDa isoform light blue arrow: 90 kDa isoform.

Cytotoxic compounds increase PTPRG release in Hep G2 cells.

Potassium dichromate is a widespread industrial waste while ethanol abuse represent a common cause of liver injury. HepG2 cells were treated in vitro with different doses of these compounds in order to evaluate whether they can modulate the release of the 120 kDa PTPRG isoform. MTT assay indicate a stronger cytotoxic effect of potassium dichromate when compared with ethanol. We measured the number of cells as calculated from the relationship among cell number/ OD (optical density at 570 nm) in MTT assay (**Fig. 12**) for correlate the OD with the cells number.

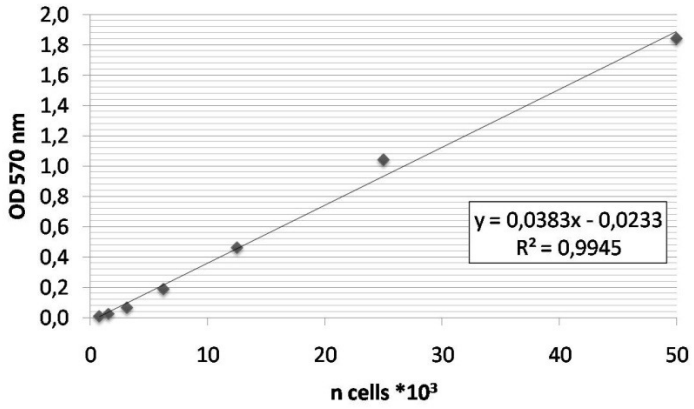
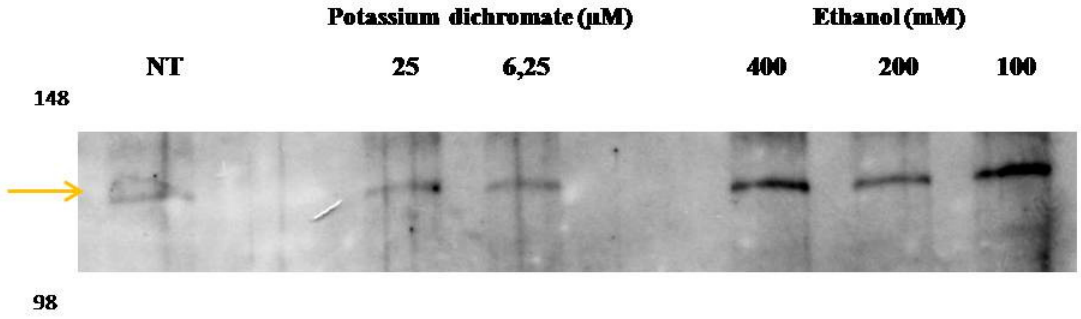


Figure 12: **MTT assay**. Correlation between the Optical density (OD) at 570 nm and cells number.

We have then quantified the amount of the 120 kDa isoform present in the conditioned medium from the same pooled wells, after 24 hours of incubation. The value was expressed as density (pixel intensity in a 256 grayscale/mm^2). Finally we correlated these values as a ratio among density of PTPRG isoform/number of live cells present in the well at the end of the experiment. Both stimuli induce the release of higher amounts of the 120 kDa isoform (2 to 7 fold) in comparison with the control (**fig 13**).



		1	2	3	4
	Concentration	**Viable cells/well (+/-SD)**	**% Cell viability**	**Density (INT/mm^2)**	**Density/cells (fold increase)**
Not treated	**0**	14732 (±1900)	**100**	3599	**0.244 (1)**
Potassium dichromate (µM)	**25**	8602 (±1000)	**56,6**	7877	**0.915 (3.7)**
	6,25	12733 (±2900)	**86,1**	6885	**0.539 (2.2)**
Ethanol (mM)	**400**	15491 (±1900)	**105,4**	15743	**1.0 (4.1)**
	200	12436 (±3200)	**83,8**	11396	**0.916 (3.7)**
	100	12491 (±3000)	**84,1**	21604	**1.729 (7)**

Figure 13: Cytotoxicyty induce the release of the extracellular PTPRG isoform in Hep G2 cells. Immunoblotting with Rb anti-P4 antibody of SFCM derived after 24 hours treatment with the indicated drugs. Column 1: number of viable cells /well (+/- standard deviation); in column 2 the % of cell viability compared to the not treated cells. In column 3 is reported the optical density (INT/mm^2) of the 120 kDa band in the different SFCM while column 4 indicate the ratio between the density and the number of viable cells (ratio among columns 3 and 1). In parentheses in column 4 is indicated the fold increase relative to the not treated sample made equal to 1.

Characterization of mAb against ECD of PTPRG

The study of a soluble form of PTPRG require the development of suitable immunodetection assays. A single monoclonal antibody (mAb) raised against the extracellular domain produced in prokaryotes (lacking the post-translational processing typical of eukaryotes) have been commercialized only very recently. We have isolated two monoclonal antibodies produced against the fully glycosilated ECD purified from eukaryotic cells.

We selected two mAb for their capability to recognize the purified PTPRG ECD by ELISA and the native antigen expressed by peripheral blood leukocytes[34] as detected by flow cytometry. In. **fig 14** a representative staining of the two mAb in comparison with the validated Ch-PTPRG antibody is shown.

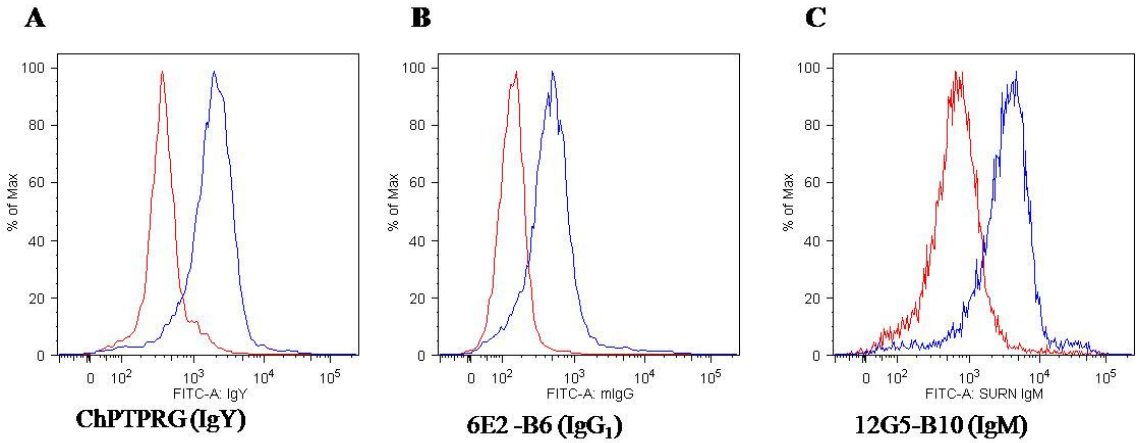


Figure 14: Flow cytometry analysis of human purified monocytes. Staining with **A**: ch-PTPRG **B**: 6E2-B6 **C**: 12G5-B10. Isotype control staining is shown in red.

To better characterize the features of these new antibodies we evaluated their capability to recognize the protein by immunoprecipitation and western blot on the recombinant extracellular domain. We analyzed the SFCM from HEK 293F cells transfected with either a PTPRG ECD cDNA truncated at the level of the putative transmembrane region linked to mouse Fc IgG_3 or with the control vector expressing only the Fc tag.

The 12G5-B10 IgM antibody specifically recognize, by western blotting, the predicted 120 kDa band in the gel lane loaded with the SFCM containing the recombinant extra cellular domain, being the supernatant derived from Fc transfected cells negative. The identity of the band was further confirmed by its reactivity with anti mouse Fc antibody (**fig 15**). In the cell lysate only the anti-Fc antibody was able to recognize the immature, not glycosylated form of the protein. This antibody does not immunoprecipitate the protein (not shown).

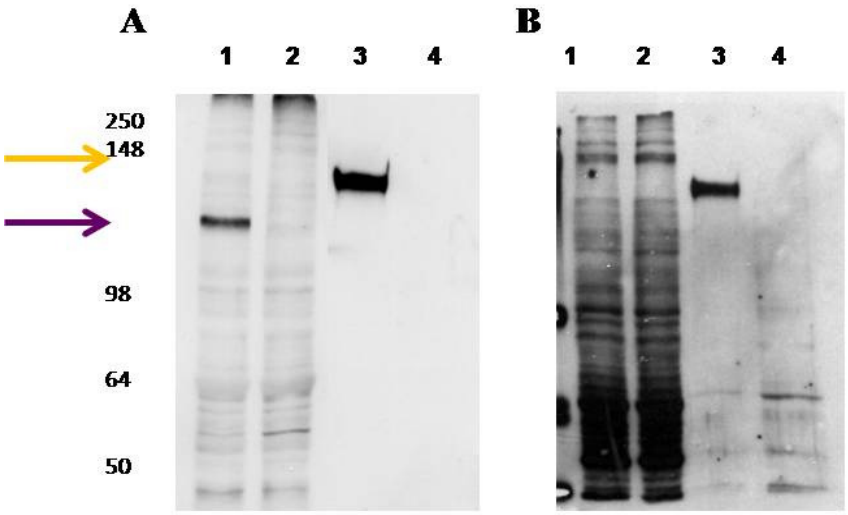


Figure 15: **Characterization of 12G5-B10 mAb**. HEK 293F cells lysate (1,2) and SFCM (3,4). Line 1,3 cells transfected with PTPRG-ECD-Fc, line 2,4 cells transfected with Fc control. **A**: WB detection with anti mouse Fc. **B**: detection with mAb anti PTPRG 12G5-B10. Yellow arrow: mature soluble ECD-Fc. Purple arrow: intracellular precursor form.

The 6E2-B6 IgG antibody is able to immunoprecipitate the mature, secreted form in SFCM and cell lysates and the immature not glycosilated form present in the cytosol **(fig 16)**

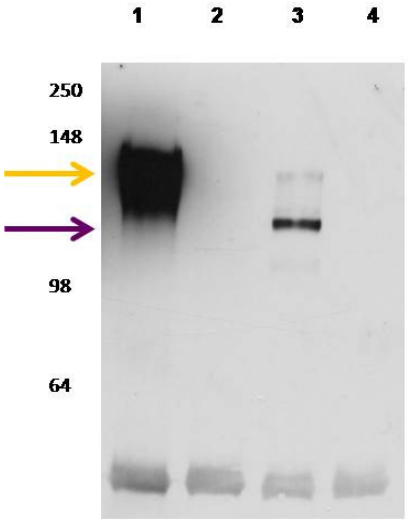


Figure 16: Characterization of 6E2-B6 IgG mAb. Immunoprecipitation of HEK 293F cells SFCM (1,2) and cell lysate (3,4) with mAb 6E2-B6 and WB detection with Rb anti-P4. Lanes 1,3 cells transfected with PTPRG-ECD-Fc, lanes 2,4 cells transfected with Fc control. Yellow arrow: mature soluble ECD-

Based on these results we conclude that 1) the 12G5-B10 IgM antibody is capable to recognize the protein by western blot and 2) 6E2-B6 IgG is capable to immunoprecipitate the native protein 3) both the antibodies can recognize the native protein by direct ELISA and by flow cytometry.

Discussion

In general terms, soluble and membrane-bound isoforms of the same protein can be generated by one of two mechanisms: 1) separate biosynthetic pathways, either by alternative pre-mRNA splicing of a common transcript or by transcription of closely related but distinct genes; 2) by posttranslational release of the extracellular domain of membrane proteins by hydrolytic cleavage of the membrane anchor. The latter appears to represent a useful and commonly employed device that allows cells to multiply the functional roles of proteins with a minimum investment in energy and structural complexity. Membrane proteins are apparently specifically released by proteolysis in a directed and sometimes regulated manner to produce active, soluble forms. These include proteins that are sufficiently diverse in structure and function to suggest that limited proteolysis may constitute a general mechanism in the release of membrane proteins to generate soluble isoforms with defined functions [38].

We have discovered the presence of a soluble PTPRG form in human and mouse plasma and serum samples. The identification of a PTPRG soluble form represent a novel finding because until now this isoform was detected only in cell culture medium of transfected cells or as mRNAs transcript in the neonatal rat brain [1]. Furthermore two glycopeptides derived from the ECD of PTPRG protein (amino acid number: 95-115 and 433-448) were identified in human serum within a large scale glycoproteomic analysis [39], thus confirming that a plasmatic form of this PTP is present.

Except from these data nothing else is present in the current scientific literature. Human blood plasma possesses significant potential for disease diagnosis and therapeutic monitoring even if it appear to be an extremely complex tissue. Indeed it appear to

contain not only “classic” plasma proteins, but also cellular “leakage” proteins that can potentially originate from virtually any cell or tissue type in the body. However, thanks to its accessibility and to the possibility to detect various kind of molecules associated to the cell metabolism, protein abundance changes in plasma may provide direct information on physiological and metabolic states of disease and drug response. As a result, the discovery of novel candidate protein biomarkers from plasma sample could open a new avenue of investigation in this challenging field.

In this study we have identified different PTPRG soluble isoforms present in human and mouse plasma including the full length PTPRG protein. Given its structural features that include a transmembrane domain, this form is probably linked to the plasma membrane associated to cell-derived exosomes. The detection of a major band of about 120 kDa protein detected with all the antibody available for PTPRG extracellular domain suggests that this isoform represent the complete ECD described by others [1]. An additional isoforms we detected include a 90 kDa protein (present in human/mouse plasma, murine liver, in the lysate and SFCM of HepG2 cell line), and a 70 kDa protein, detected in all the perfused tissues analyzed.

Taking advantage by the use of a large series of antibodies, most of which developed in our laboratory, we can conclude that the 90 kDa protein may be missing a part of the N-terminal of the ECD and possess a short tail of the intracellular domain, as it reacts with an antibody raised against an epitope located in this region. A possibility exists that 120 kDa protein and the 90 co-immunoprecipitate under the experimental conditions used in our assays. This last interpretation is suggested by the observation that the association of E and P subunits of PTPRF (LAR), not disulfide bonded, was stable under standard cell lysis condition and was sufficiently strong to resist disruption during vigorous washing of the immunoprecipitates [25]. Also PTPRS was expressed in two subunits that

were derived from a precursor protein by proteolytic processing and also in this case E and P subunits co-imunoprecipitate [40]. The visualization of the 120 and 90 kDa isoforms in HepG2 cell line and in liver of perfused mice support the hypothesis that the liver produces a soluble form of extracellular domain that is released in plasma of healthy donor. The analysis of PTPRG expression in organs highlight the presence of a 70 kDa band that might represent an immature deglycosylated form of the soluble protein or a fragment of the 120 kDa isoform lacking part of the C-terminal domain of the ECD.

We now need to correlate ECD relese to a specific function and/or physiophatological condition. In order to start to address this issue we first wonder whether cytoxicity could alter the release of this protein in the extracellular space. For this purpose we used the Hep G2 cell model and observed that potassium dichromate and ethanol induced an increased amount of the released 120 kDa isoform.

The question whether the soluble form of PTPRG could reflect a specific physiopathological condition require the study of a large series of clinical information-rich plasma samples and the development of medium-high throughput screening assays. One of the most widely utilized in research and clinical biochemistry, is represented by ELISA assays. Unfortunately this assay is not currently available for this protein for the absence of suitable antibodies. Given this premise, we decided to develop new reagents capable to react against the native form of this PTP in the form of monoclonal antibodies.

The immunization procedure gave rise to several clones, two of which were selected for further characterizaton. 6E2-B6 and 12G5-B10 recognize the native protein by ELISA and flow cytometry assays. In addition, 6E2-B6 mAb was capable to recognize the native, full-length PTPRG protein by immunoprecipitation. The mAb 12G5-B10

specifically recognized the eukaryotic recombinant ECD expressed in HEK 293F cells by western blotting but could not detect the unprocessed cytoplasmic form in the same cells. This could be related to a lower affinity for this antigen or the different protein folding patterns, since immature PTPRG proteins could result in the exposure of unique immunogenic epitopes different from those present in the folded proteins. An alternative explanation, that we favor as the antibody recognize the fully processed protein released in the extracellular space, is that the epitope recognized by this antibody is glycosilated. Future experiments will better address this issue.

In summary, we generated two new mAbs that may provide suitable reagents for further investigation of the functional role of PTPRG proteins and open the possibility to quantify the native protein in cells and biological fluids derived from patients. This, in turn, will provide the necessary informations to unravel the role of sPTPRG in health and disease.

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