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Protein Tyrosine Phosphatase Receptor Type γ Is a JAK Phosphatase and Negatively Regulates Leukocyte Integrin Activation

Michela Mirenda,* Lara Toffali,*† Alessio Montresor,*† Giovanni Scardoni,† Claudio Sorio,* and Carlo Laudanna*§†

Regulation of signal transduction networks depends on protein kinase and phosphatase activities. Protein tyrosine kinases of the JAK family have been shown to regulate integrin affinity modulation by chemokines and mediated homing to secondary lymphoid organs of human T lymphocytes. However, the role of protein tyrosine phosphatases in leukocyte recruitment is still elusive. In this study, we address this issue by focusing on protein tyrosine phosphatase receptor type γ (PTPRG), a tyrosine phosphatase highly expressed in human primary monocytes. We developed a novel methodology to study the signaling role of receptor type tyrosine phosphatases and found that activated PTPRG blocks chemoattractant-induced β2 integrin activation. Specifically, triggering of LFA-1 to high-affinity state is prevented by PTPRG activation. High-throughput phosphoproteomics and computational analyses show that PTPRG activation affects the phosphorylation state of at least 31 signaling proteins. Deeper examination shows that JAKs are critically involved in integrin-mediated monocyte adhesion and that PTPRG activation leads to JAK2 dephosphorylation on the critical 1007–1008 phosphotyrosine residues, implying JAK2 inhibition and thus explaining the antiadhesive role of PTPRG. Overall, the data validate a new approach to study receptor tyrosine phosphatases and show that, by targeting JAKs, PTPRG downmodulates the rapid activation of integrin affinity in human monocytes, thus emerging as a potential novel critical regulator of leukocyte trafficking. The Journal of Immunology, 2015, 194: 2168–2179.

Integrin activation is a critical step in leukocyte recruitment (1). A number of protein and lipid kinases have been shown to regulate the proadhesive signaling network triggered by chemoattractants (2). In this context, we have recently shown that protein tyrosine kinases (PTKs) of the JAK family are upstream transducers linking the chemokine receptor CXCR4 to the hierarchical activation of Rho and Rap small GTPases, thus controlling integrin affinity upregulation and homing to secondary lymphoid organs of T lymphocytes (3). Overall, although we are still far from a complete characterization of this complex signaling mechanism, it is clear that leukocyte trafficking is under tight control of phosphorylating events. Notably, leukocyte adhesion is a transient phenomenon showing oscillating dynamics, cycling between adhesion and de-adhesion states propaedeutic to cell crawling, diapedesis, and chemotaxis. However, although negative regulators of integrin activation have been described, the mechanisms regulating the on–off dynamics of kinase activity controlling integrin triggering by chemoattractants are unknown. Thus, considering the proadhesive role of JAKs, it is logical to expect that downmodulation of JAK activity could affect leukocyte adhesion dynamics. This may suggest the possibility that specific protein tyrosine phosphatases could be involved in the overall process of chemoattractant-induced leukocyte recruitment.

The protein tyrosine phosphatases (PTP) superfamily includes receptor-like PTP (RPTP) and nontransmembrane proteins for a total of 38 coding genes (4, 5). Particularly, RPTPs display a modular structure including a variable extracellular region, consisting of different domains possibly implicated in cell–cell and cell–matrix adhesive contacts, and an intracellular region commonly shared with other components of the superfamily. The intracellular region is typically composed of two domains named D1 and D2 (intracellular domain [ICD]). The catalytic activity resides in the D1 domain, whereas the D2 domain is possibly involved in substrate specificity, stability, and protein–protein interaction of RPTPs. The activity of RPTPs is controlled by a variety of mechanisms (5). Although not established for all RPTPs, the main regulatory mechanism of RPTP activity consists of the reversible transition from a homodimeric inactive form to a monomeric active form (6, 7). Oxidative stress is also an important regulatory mechanism of RPTPs, leading to homodimer inactivation and hence to the inactivation of the enzyme itself (4).

The lack of techniques allowing specific upmodulation of PTP activity in primary cells has hampered investigating the regulatory implications of tyrosine phosphorylation/dephosphorylation turn-over under physiological conditions. Notably, it cannot be assumed a priori whether tyrosine dephosphorylation plays, at the cellular level, positive or negative regulatory roles, making unclear which is the best approach to study tyrosine phosphatases under...
physiological conditions. For instance, in a context where PTK activity mediates a positive regulation, such as the role of JAKs in integrin activation, an experimental approach based on chemical PTP-specific inhibitors or small interfering RNA silencing could be misleading. Alternatively, methods allowing selective activation of PTPs in primary cells are unavailable. To investigate the regulation of tyrosine dephosphorylation in chemoattractant-triggered signal transduction in human leukocytes, we developed a novel approach based on cell-penetrating peptides (CPPs) and allowing studying the effect of PTP receptor type γ (PTPRG) activation in human primary monocyte adhesion. We demonstrate that specific activation of PTPRG tyrosine phosphatase activity is achieved by means of two complementary CPP approaches leads to inactivation of LFA-1 high-affinity-state triggering and mediated underflow cell arrest by chemoattractants. We show that this effect is accompanied by a consistent remodeling of signaling phosphonetwork triggered by chemoattractants. More specifically, PTPRG activation leads to JAK2 dephosphorylation and inhibition, thus showing that JAK2 are targets of PTPRG. These findings show that PTPRG is a novel critical regulator of leukocyte trafficking. More generally, the data describe a new approach suitable to study the functional interplay between tyrosine kinase and phosphatase activities under physiological conditions.

Materials and Methods

Human primary cells and reagents

Informed consent for this work was provided by the University of Verona; the University of Verona Ethics Committee approved experimentation involving human primary cells. Human primary monocytes and polymorphonuclear cells were isolated from whole blood of healthy donors. Purity of monocyte preparation was evaluated by flow cytometry after staining with FITC-conjugated anti-CD14 Ab and was >95%. Isolated cells were kept at 37°C in standard adhesion buffer (PBS, 1 mM CaCl2, 1 mM MgCl2, 10% FBS [pH 7.2]) and used within 1 h. FBS was from Lonza; fMLF, human fibrinogen, ICAM-1, and E-selectin were from R&D Systems; FITC-conjugated goat to mouse Ab was from Sigma-Aldrich; the nAb 327C detecting an LFA-1 epitope corresponding to high-affinity state was provided by Dr. Kristine Kikly (El Lilly and Co.); tyrophostin AG940 was from Sigma-Aldrich; WHI-P154 was from Calbiochem; the monoclonal anti-human PTPRG Ab was from R&D Systems; rabbit monoclonal anti-JAK2 (D2E12) was from Cell Signaling Technology; rabbit monoclonal anti-phospho-JAK2 (E132) was from EMD Millipore; anti–phospho-JAK2 was provided by Dr. Dr. A.W. Segal (Department of Medicine, University College, London, U.K.) (8); 4-nitrophenyl phosphate disodium salt hexahydrate was from Sigma-Aldrich.

Trojan CPP technology

The control TAT and Penetratin 1 (P1) peptides and the fusion P1-PTPRG wedge domain (P1-WD), P1-PTPRG wedge scrambled (P1-WD_scrambled), and P1-JAK2 (P1–tyrosine kinase inhibitor peptide [TKIP]) peptides were synthesized by GenScript.

The P1-WD peptide (RQIKIWFQNRRMKWKKGKQFVKHI) encompassed the complete P1 sequence (16 aa; RQIKIWFQNRRMKWKK, an inserted glycine to allow flexibility of the fusion peptide, and the TKIP sequence (12 aa; WLVFFAFYFPR), specifically blocking JAK2 autophosphorylation (3). The P1-TKIP peptide displayed the following properties: amino acids, 29; molecular mass, 3069.89 Da; isoelectric point, 12.02 (as calculated by EMBoss pepstats computation, http://www.ebi.ac.uk/Tools/seastats/emboss_pepstats/). Peptide stock solutions (10 mM) in DMSO were kept at −80°C and diluted in adhesion buffer immediately before the experiments. All peptides were fully soluble in aqueous buffers. Standard treatment of cells with peptides was for 1 h at 37°C in 24- or 6-well plates.

PTPRG TAT-ICD, TAT-ICD_D1028A, and TAT-enhanced GFP (eGFP) fusion proteins were generated by using the pRES2-TATA expression vector, specifically designed to allow protein in-frame expression of TAT fusion proteins, as we previously described (10). Briefly, the cDNAs encoding for the complete ICD of PTPRG (aa 797–1445) and for the complete eGFP sequence were cloned into pRES2-TATA vector between BamHI and EcoRI sites. The ICD_D1028A cDNA was obtained by site-directed mutagenesis, wherein the Asp1028 codon was replaced with an Ala codon and verified by sequencing. Expression of recombinant TAT-ICD, TAT-ICD_D1028A, and TAT-eGFP fusion proteins was controlled to allow purification under nondenaturing conditions. Overnight cultures of the Escherichia coli strain BL21 (DE3) plxS harboring recombinant plasmid expressing (His)6-TAT-ICD, (His)6-TAT-ICD_D1028A, and (His)6-TAT-eGFP were diluted in fresh Luria–Bertani medium containing ampicillin (50 μg/ml) and chloramphenicol (35 μg/ml) and grown at 37°C to an OD600 of 0.6. Protein expression was induced overnight at 16°C with 0.1 mM isopropyl β-D-thiogalactoside. Harvested cells were resuspended in 1:50-culture volume of lysis buffer containing 20 mM tris, 300 mM NaCl, 10 mM imidazole [pH 7.3], and protease inhibitors without EDTA. Samples were sonicated five times for 30 s at 4°C and centrifuged for 15 min in a Sorval SS34 rotor at 12,500 rpm at 4°C. After centrifugation, the supernatants were 0.45-μm porous-filtered and quantified by Bradford assay. By SDS-PAGE containing 100 mM DTT, 200 μg/ml, and 10 mM DTT, and 200 μg/ml (MILLiPORE) followed by a buffer exchange in storage buffer containing 100 mM arginine, 20 mM Tris, and 150 mM NaCl. Concentrated stock solutions of purified native TAT fusion proteins were stored at −20°C. The tyrosine phosphatase activity of the TAT-ICD preparations was systematically quantified by means of a p-nitrophenyl phosphate (pNPP) assay. Freshly purified TAT-ICD was dissolved in pNPP assay buffer (20 mM Tris, 10 mM DTT, and 2 mM pNPP) in the presence or absence of orthovanadate in the absence of orthovanadate and the dephosphorylating activity was evaluated, after 30 min of incubation, at 405 nm by a plate reader (Victor X5 multilabel plate reader, PerkinElmer). Standard treatment of cells with 0.22-μm porous-filtered TAT-ICD was for 60 min at 37°C in 24- or 6-well plates.

Membrane fraction isolation and pNPP assay

Freshly isolated monocytes and polymorphonuclear cells (PMNs) were suspended in 100 mM PIPES, 12.5 mM EGTA, 300 mM KCl, 30 mM NaCl, and 35 mM MgCl2, (pH 7.3) (relaxation buffer) in the presence of protease inhibitors. Cells were then sonicated twice (6 s, 15 W) and centrifuged at 2000 rpm for 10 min. The supernatants were collected and centrifuged at 28,000 rpm for 60 min. After centrifugation, the supernatants were discarded and the membrane pellets were dissolved in 20 mM Tris (pH 7.4). Equal amounts of membrane fractions were added to pNPP buffer containing 20 mM tris, 10 mM DTT, and 2 mM pNPP, after which DMSO and peptides were added. After 30 min of incubation, levels of dephosphorylation, in the presence or absence of orthovanadate, were measured at 405 nm by a plate reader (Victor X5 multilabel plate reader, PerkinElmer).

Western blot

Cells were lysed in ice-cold 1% Nonidet P-40 buffer containing phosphatase inhibitors and complete protease inhibitor mixture (Roche). Total cell lysate (100 μg) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted. The following proteins were subjected to 10% SDS-PAGE and then blotted. After incubation with anti–PTPRG, anti–phospho-JAK2, anti-JAK2, and HRP-linked secondary Abs (GE Healthcare), immunoreactive bands were visualized by ECL detection (EMD Millipore). Intensities of band signals were quantified by densitometric analysis (Quantity One, Bio-Rad) by using ImageQuant Las4000.

Static adhesion assay

Human primary monocytes or PMNs were suspended at 5 × 106/ml in standard adhesion buffer. Adhesion assays were performed on 18-well
PTPRG NEGATIVELY REGULATES MONOCYTE ADHESION

Glass slides coated with 1 mg/ml human fibrinogen or with 1 µg/ml human ICAM-1 in PBS, 20 µl cell suspension was added to the coated wells and stimulated for 1 min at 37°C with 5 µl IMLF, 0.05 µM final concentration. After rapid washing, adherent cells were fixed in ice-cold 1.5% glutaraldehyde in PBS, and still images of adherent cells in 0.2-mm² fields were acquired at ≥20 phase contrast magnification (numerical aperture, 0.40) with a charge-coupled device camera (ICD-42B, Ikegami) connected to an inverted microscope (IX50; Olympus). Image acquisition and computer-assisted enumeration of adherent cells were performed with ImageJ (National Institutes of Health).

Underflow adhesion assay

Human primary monocytes were suspended in 1 × 10⁷/ml in standard adhesion. Cell behavior in underflow conditions was analyzed with the BioFlux 200 system (Fluxion Biosciences). Forty-eight–well plate microfluidics were first coated overnight at room temperature with 2.5 µg/ml human E-selectin and 5 µg/ml human ICAM-1 in PBS. Immediately before use, microfluidic channels were washed with PBS and then coated with 10 nM IMLF in PBS for 3 h at room temperature. After extensive washing of microfluidics with adhesion buffer, cells were fluxed at 2 dynes/cm² wall shear stress and the behavior of interacting cells was assessed from human primary PMNs. Western blot analysis showed that PTPRG is expressed in monocytes but absent in PMNs (19) (see also http://www.phosphosite.org/). Phospho-ELM (http://phospho.elm.eu.org), PhosphoNET (http://www.phosphonet.ca), and KEA (http://amp.pharm.mssm.edu/keablast) were previously normalized to control cells and statistically analyzed (see Materials and Methods), thus suggesting the capability of this region to support phosphatase activity by removing intermolecular D2 versus D1 phosphatase activity on cell plasma membrane as a phosphatase-inactive homodimer. Therefore, we reasoned that interference by steric hindrance with the WD could potentially lead to PTPRG monomerization and/or catalytic domain separation, thus freeing the intrinsic tyrosine phosphatase activity by removing intermolecular D2 versus D1 domain inhibition (15). Bioinformatics analysis based on comparison with previously characterized WDs in PTPRA (14), PTP receptor type C (PTPRC) (6), LAR (16), and PTPRM (16), performed with BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) and PTP-dedicated online resources (http://ptp.cshl.edu), identified the PTPRG WD as a juxtamembrane 26-aa sequence enriched with tyrosine kinase motifs. Notably, previous data suggest a unique capability of PTPRG intracellular domains to undergo in vitro dimerization (15), thus suggesting that PTPRG is possibly expressed on cell plasma membrane as a homo- or heterodimer. Under a helix-turn-helix structure (Fig. 1B), and a high solvent accessibility, as evidenced by residue analysis and hydrophathy calculation and by accessible surface area (9) calculations (see Materials and Methods), thus suggesting the capability of this region to support protein interactions. We first tested the biochemical activity of the fusion peptide by performing in vitro assays of phosphatase activity on purified membranes isolated from human primary monocytes compared with membranes isolated from human primary PMNs. Western blot analysis showed that PTPRG is expressed in monocytes but absent in PMNs (Fig. 1C) (see also previously suggested (19)) (see also http://humanproteome.org/). Notably, Western blot of total monocyte lysates reveals a double PTPRG band absent in the membrane fraction, likely due to differential PTPRG posttranslational mod-

PTPRG negatively regulates monocYTE adhEsion

Results

Identification and functional characterization of the PTPRG WD

To develop a methodology allowing modulation of PTPRG enzymatic activity in primary leukocytes, we exploited some properties of RPTPs. Although variability may occur among the different RPTP subgroups (5), the inhibitory receptor homodimerization has been suggested to involve, under physiological conditions, a region, called WD, partially conserved between the different RPTPs and involved in intracellular domain interaction and consequent enzymatic inhibition of the D1 catalytic domain (6, 14). Notably, previous data suggest a unique capability of PTPRG intracellular domains to undergo in vitro dimerization (15), thus suggesting that PTPRG is possibly expressed on cell plasma membrane as a phosphatase-inactive homodimer. Therefore, we reasoned that interference by steric hindrance with the WD could potentially lead to PTPRG monomerization and/or catalytic domain separation, thus freeing the intrinsic tyrosine phosphatase activity by removing intermolecular D2 versus D1 domain inhibition (15). Bioinformatics analysis based on comparison with previously characterized WDs in PTPRA (14), PTP receptor type C (PTPRC) (6), LAR (16), and PTPRM (16), performed with BLASTp (http://www.ncbi.nlm.nih.gov/Tools/sssb/ncbiblast/) and PTP-dedicated online resources (http://ptp.cshl.edu), identified the PTPRG WD as a juxtamembrane 26-aa sequence encompassing aa 831–856, with the last 11 aa belonging to the D1 phosphatase-active domain (Fig. 1A). The PTPRG WD displays a helix-turn-helix structure (Fig. 1B) and a high solvent accessibility, as evidenced by residue analysis and hydrophathy calculation and by accessible surface area (9) calculations (see Materials and Methods), thus suggesting the capability of this region to support protein interactions. A fusion PI-WD Trojan peptide (17, 18) was then generated (see Materials and Methods) to allow overstepping cell plasma membrane, thus investigating the biological activity of the PTPRG WD peptide in primary cells. We first tested the biochemical activity of the fusion peptide by performing in vitro assays of phosphatase activity on purified membranes isolated from human primary monocytes compared with membranes isolated from human primary PMNs. Western blot analysis showed that PTPRG is expressed in monocytes but absent in PMNs (Fig. 1C), as also previously suggested (19) (see also http://humanproteome.org/). Notably, Western blot of total monocyte lysates reveals a double PTPRG band absent in the membrane fraction, likely due to differential PTPRG posttranslational mod-

JAK2 activation assay

JAK2 activation was determined with ELISA kit JAK2 (pYpY1007/1008) (Invitrogen). Briefly, cells were stimulated with agonist and lysed following the manufacturer’s protocol; levels of phospho-JAK2 were quantified (absorbance at 450 nm) with a microwell plate reader (Victor X5 multi-label plate reader, PerkinElmer).

Protein–protein interaction assay

His-Select nickel affinity gel (30 µl; Sigma-Aldrich), previously washed three times with TBS and with bacterial lysis buffer, was incubated for 3 h at 4°C with 3 mg quantified supernatants expressing Tat-ICD_D1028A or Tat-eGFP under constant rotation. After 3 h, the nickel affinity gel was washed three times with monocyte lysis buffer (previously described). Each reaction, containing immobilized Tat-ICD_D1028A or Tat-eGFP in 30 µl His-Select nickel affinity gel, was incubated with 500 µg monocyte lysate treated with buffer (control) or with 50 nM IMLF for 3 h at 4°C with constant rotation. After incubation, beads were washed at least three times in monocyte lysis buffer and in TBS. Proteins were eluted from the beads in SDS sample buffer and resolved by SDS-PAGE and Western blot analysis (13).

Statistical analysis

Statistical analysis was performed by calculating means and SD from different experiments; significance was calculated by ANOVA followed by a Dunnett post hoc test versus control condition indicated in each graph.
ification, such as glycosylation (20). In contrast, other membrane markers, such as CD45 (PTPRC), gp91phox (NADPH oxidase component), and CD18 were correspondingly expressed in monocytes as well as in PMNs (not shown). The P1-WD peptide elicited in a dose-dependent manner a consistent phosphatase activity in membrane isolated from monocytes (Fig. 1D). In contrast, control P1 and P1-WD_scrambled peptides were minimally effective. Orthovanadate treatment completely prevented phosphatase activity, supporting the triggering of a tyrosine phosphatase activity (not shown). Furthermore, and very importantly, the P1-WD peptide did not elicit a consistent phosphatase activity in membrane isolated from PMNs (Fig. 1E). Altogether, these data corroborate our hypothesis, suggesting that the P1-WD peptide may act as an activator of PTPRG tyrosine phosphatase activity, possibly by promoting PTPRG monomerization and D1 domain catalytic activity.

Activation of PTPRG inhibits LFA-1 affinity triggering and mediated adhesion by chemoattractants in human primary monocytes

We then investigated the functional effect of PTPRG activation on integrin activation and mediated adhesion by chemoattractants in human primary monocytes. Treatment of monocytes with the P1-WD peptide prevented in a dose-dependent manner fMLF-triggered static adhesion to fibrinogen (Fig. 2A) as well as to ICAM-1 (Fig. 2B), thus suggesting blockade of Mac1 (CD11b/CD18), P150/95 (CD11c/CD18), and LFA-1 (CD11a/CD18) β2 integrin activation. The P1-WD peptide also prevented monocyte arrest on ICAM-1 in underflow adhesion assays (Fig. 1C), a condition dependent on rapid LFA-1 triggering to a high-affinity state (10, 21, 22). Indeed, treatment with P1-WD led to a consistent reduction of arrested cells accompanied by a corresponding increase of rolling cells, as expected. This finding was confirmed in LFA-1 affinity assays by using a specific reporter mAb detecting a fully extended LFA-1 conformational epitope corresponding to heterodimer high-affinity state. Pretreatment with P1-WD led to a very consistent inhibition of LFA-1 triggering to high-affinity state by fMLF (Fig. 1D). In the assays, treatment with the P1 or P1-WD_scrambled peptides was completely ineffective, thus supporting the specificity and excluding toxic effects of the P1-WD peptide. Importantly, PMNs were completely unaffected by P1-WD treatment (not shown). Taken together, these data strongly suggest that activated PTPRG acts as a negative regulator of signaling mechanisms controlling integrin activation and mediated adhesion in human primary monocytes. To confirm these findings with a complementary approach, we exploited a different Trojan peptide approach based on a TAT fusion protein technology we previously developed (10). A fusion recombinant protein was generated encompassing the complete PTPRG intracellular phosphatase domains (ICD, aa 846–1407), including both D1 and D2 domains (Fig. 1A), fused, at the N-terminus, to the Trojan 13-aa TAT sequence, capable of transporting biologically active proteins into the cell (17). PTPRG TAT-ICD fusion protein retained tyrosine phosphatase activity, as verified in in vitro phosphatase assays in the absence and presence of orthovanadate (Fig. 3A), thus showing the presence of soluble, catalytically active, TAT-ICD monomers. We then investigated the functional effect of PTPRG TAT-ICD on integrin activation and mediated adhesion by chemoattractants. Monocyte treatment with TAT-ICD

FIGURE 1. The P1-WD peptide triggers a specific tyrosine phosphatase activity in human monocyte membranes. (A) Diagram of PTPRG protein domains. (B) Crystallographic renderings of PTPRG D1 catalytic and wedge (red) domains (14–16). (C) Western blot of PTPRG expression in monocytes and PMNs. PTPRG expression was assessed in total cell lysates and membrane fractions with a mouse monoclonal anti-PTPRG Ab. Whole-cells lysates were probed with rabbit anti-actin Ab, whereas membrane fraction lysates were probed with rabbit anti-gp91phox as loading controls; a representative experiment of three independent experiments is shown. (D) pNPP assay on human monocyte and (E) PMN membranes. Isolated membranes were dissolved in pNPP buffer and treated with DMSO, with the indicated concentrations of P1-WD or with 200 μM P1 or P1-WD_scrambled peptides. Values are absorbance at 405 nm shown as percentage fold increase (FI) over the blank; mean values of three experiments are shown; error bars are SDs. *p < 0.05, ***p < 0.001 versus vehicle.
The previous data imply PTPRG as a downregulator of chemoattractant signaling. To identify PTPRG targets in the context of fMLF signaling, we employed a high-throughput Ab array technology. Notably, although the two Trojan peptide approaches provided fully coherent functional results, the P1-WD approach is potentially more specific than the TAT-ICD–based one. Indeed, the catalytic domains of many RPTPs appear extremely promiscuous compared with respect to substrate specificity (15). Moreover, sequence alignment shows more variability in the WDs versus D1/D2 domains, which appear more conserved (see http://ptp.cshl.edu). Consistently, the P1-WD did not elicit a relevant tyrosine phosphatase activity in membrane isolated from PMNs, which do express CD45 (PTPRC) but not PTPRG. These considerations may suggest that modulation of PTPRG tyrosine phosphatase activity by means of P1-WD peptide may be a more specific approach to investigate the influence of PTPRG on signaling tyrosine phosphonetworks. Thus, we focused the phosphoproteomics analysis on the effect of P1-WD. We also focused on fMLF-potentiated signaling networks, because this directly correlates with integrin triggering and mediated rapid adhesion. PTPRG activation by the P1-WD peptide affected the tyrosine phosphorylation of several signaling proteins. Data analysis identified 31 molecules whose phosphorylation was modified in a statistically significant manner (Table I). Interestingly, 16 proteins showed reduced tyrosine phosphorylation, possibly indicating a direct PTPRG targeting activity. In contrast, 15 proteins showed an increased tyrosine phosphorylation. This may suggest an indirect and more complex effect, possibly mediated by PTPRG-sensitive, concurrent, signaling mechanisms, thus indicating that PTPRG, beside a dephosphorylating activity on direct targets, may also generate nonlinear effects mediated by more complex signaling cascades. Literature data mining suggests that the functional activity of a subgroup of 16 proteins, including ABL1, BMX, BTK, CDK2, CTNN, DAB1, ITGB1, JAK2, KDR, KIT, LIMK1, MET, PDGFRB, SHC1, SRC, and VCL, is inhibited by PTPRG activation. Among these, inhibition of ABL1, BMX, BTK, DAB1, ITGB1, JAK2, KDR, KIT, LIMK1, MET, PDGFRB, SHC1, and VCL correlates with tyrosine dephosphorylation. In contrast, SRC inhibition correlates with hyperphosphorylation of the inhibitory Tyr530 residue and with dephosphorylation of the activatory Tyr419. Moreover, CDK2 and CTNN inhibition correlates with a hyperphosphorylation of the inhibitory Tyr15 and Tyr470, respectively. In contrast, a subgroup of 13 proteins, including BLNK, DOK2, ERBB2, GRIN2B, INSR, PDGFR, PRKCD, PXN, STAT1, STAT2, STAT3, STAT5A, and ZAP70, appears to be activated by PTPRG activity. Among these, activation of BLNK, DOK2, ERBB2, GRIN2B, INSR, PDGFR, PRKCD, PXN, STAT1,
The high-throughput phosphoproteomics analysis showed that PTPRG activation leads to JAK2 protein tyrosine kinase dephosphorylation (Fig. 4, Table I). These data attracted our attention because JAKs regulate, in human primary T lymphocytes, LFA-1 and VLA-4 activation by chemokines and mediated trafficking to secondary lymphoid organs (3). Thus, JAK2 dephosphorylation by PTPRG could explain, at least partially, the antiadhesive role of PTPRG on monocytes. However, the role of JAKs in integrin activation in primary monocytes is unknown. Furthermore, the capability of IMLF to trigger JAKs activation was never addressed. Thus, we first set out to define the role of JAK2 in fMLF-triggered rapid adhesion in monocytes. Time course experiments, either performed with ELISA assay (Fig. 5A) or Western blot (Fig. 5B, 5C), showed that JAK2 is effectively phosphorylated and activated in monocytes stimulated by fMLF. Western blot (Fig. 5B, 5C), showed that JAK2 is effectively phosphorylated and activated in monocytes stimulated by fMLF. To evaluate whether JAK2 mediates fMLF-triggered rapid adhesion in human monocytes, we tested the capability of three JAK inhibitors, AG490, WHI-P154, and P1-TKIP peptide, to block adhesion triggering. All of the inhibitors completely prevented JAK2-mediated fMLF-induced adhesion in monocytes. Thus, we first set out to define the role of JAK2 in fMLF-triggered rapid adhesion in monocytes. Time course experiments, either performed with ELISA assay (Fig. 5A) or Western blot (Fig. 5B, 5C), showed that JAK2 is effectively phosphorylated and activated in monocytes stimulated by fMLF. To evaluate whether JAK2 mediates fMLF-triggered rapid adhesion in human monocytes, we tested the capability of three JAK inhibitors, AG490, WHI-P154, and P1-TKIP peptide, to block adhesion triggering. All of the inhibitors completely prevented fMLF-induced tyrosine autophosphorylation and activation of STAT1, EGFR, PDGFRB, ABL1, and BTK (Supplemental Table I), suggesting that, in the context of chemoattractant signaling, Src family kinases are possibly main functional targets of PTPRG activity. Network modular decomposition (28–30) allowed isolating two modules, prevalently including activated PTK2 and EGFR, functional interpretation of phosphotyrosine status was ambiguous. Network inference analysis shows that the 31 identified proteins generate a single network-connected component, with no isolated nodes, suggesting that PTPRG may affect the function of an integrated signaling macromodule controlling many cellular functions (Fig. 4A). Graph topological analysis performed by applying mathematical algorithms computing network node centrality indexes, categorizing proteins by topological relevance (11, 23–26), and predicting functional significance (27) shows that SRC appears by far the most connected and central signaling protein in the network, followed by KIT, PTK2, ITGB1, STAT1, EGFR, PDGFRB, ABL1, and BTK (Supplemental Table I), suggesting that, in the context of chemoattractant signaling, Src family kinases are possibly main functional targets of PTPRG activity. Network modular decomposition (28–30) allowed isolating two modules, prevalently including activated (Fig. 4B) or inhibited proteins (Fig. 4C). Thus, the two modules may potentially represent a more specific and structured context of functional influence of PTPRG on chemoattractant signaling in human monocytes. Notably, the graph edge directionality also highlights the signaling flow in the modules, thus facilitating the inference of protein reciprocal functional influence.

Overall, the combined analysis shows that PTPRG activation may determine a complex remodeling of chemoattractant-triggered signaling network. The protein kinases SRC, KIT, ABL1, ZAP70, BTK, CDK2, JAK2, KDR, PDGFRA, PDGFRB, PRKCD, and PTK2 appear as direct or indirect PTPRG targets, possibly resulting in inhibition (SRC, KIT, ABL1, BTK, CDK2, JAK2, KDR, PDGFRA) or activation (ZAP70, PDGFRA) of their functional activities.

**JAK2 mediates LFA-1 affinity triggering and mediated adhesion by chemoattractants in human primary monocytes**

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**FIGURE 3.** The TAT-ICD fusion protein inhibits LFA-1 affinity triggering and mediated adhesion by chemoattractants in human primary monocytes. (A) pNPP assay with 50 μM TAT control peptide or with 50 μM TAT-ICD in the presence or absence of 200 μM NaN3VO4. Substrate dephosphorylation was detected by reading the absorbance at 405 nm after 30 min of incubation. Shown is percentage fold increase over the pNPP buffer; mean values of seven experiments in triplicate are shown; errors bars are SDs. Static adhesion assays on (B) fibrinogen or (C) ICAM-1 are shown. Human monocytes were treated with buffer (control) or the indicated concentrations of TAT-ICD or TAT peptide for 1 h at 37˚C and stimulated with 50 nM fMLF for 60 s; mean values of five experiments in triplicate are shown; errors bars are SDs. (D) Underflow adhesion assay. Human monocytes were treated with buffer (control), 50 μM TAT peptide, or 0.5 μM TAT-ICD for 1 h at 37˚C. Shown are percentages of rolling and arrested cells for the indicated times over total interacting cells; mean values of four experiments are shown; errors bars are SDs. (E) LFA-1 high-affinity-state detection. Human monocytes were treated with buffer (control), 50 μM TAT peptide, or 0.5 μM TAT-ICD for 1 h at 37˚C and stimulated with 50 nM IMLF for 60 s. LFA-1 high-affinity-state conformer was detected by cytofluorimetric analysis with the 327C Ab. Shown are percentages of fold increases over the control; mean values of six experiments are shown; errors bars are SDs. **p < 0.01, ***p < 0.001 versus CTRL or TAT. CTRL, control.
In this study we examined the role of RPTPs in the regulation of leukocyte recruitment. We focused the analysis on PTPRG and on human primary monocytes triggered to LFA-1 high-affinity-state upregulation by the FPR1 ligand fMLF. To investigate the functional effect of PTPRG activation, we developed a CPP-based approach allowing testing in primary cells the impact of PTPRG activity on chemoattractant-triggered signaling networks. The results can be summarized as follows: 1) a combined CPP-based approach is suitable to study the regulatory role of PTPRG activation on signal transduction in primary cells; 2) PTPRG appears expressed in primary monocytes as an inactive homodimer, whose catalytic activity can be upregulated by interfering with the putative functional effect of PTPRG activation, we developed a CPP-based approach allowing testing in primary cells the impact of PTPRG activity on chemoattractant-triggered signaling networks. The results can be summarized as follows: 1) a combined CPP-based approach is suitable to study the regulatory role of PTPRG activation on signal transduction in primary cells; 2) PTPRG appears expressed in primary monocytes as an inactive homodimer, whose catalytic activity can be upregulated by interfering with the putative functional effect of PTPRG activation, we developed a CPP-based approach allowing testing in primary cells the impact of PTPRG activity on chemoattractant-triggered signaling networks. The results can be summarized as follows: 1) a combined CPP-based approach is suitable to study the regulatory role of PTPRG activation on signal transduction in primary cells; 2) PTPRG appears expressed in primary monocytes as an inactive homodimer, whose catalytic activity can be upregulated by interfering with the putative functional effect of PTPRG activation, we developed a CPP-based approach allowing testing in primary cells the impact of PTPRG activity on chemoattractant-triggered signaling networks. The results can be summarized as follows: 1) a combined CPP-based approach is suitable to study the regulatory role of PTPRG activation on signal transduction in primary cells; 2) PTPRG appears expressed in primary monocytes as an inactive homodimer, whose catalytic activity can be upregulated by interfering with the putative functional effect of PTPRG activation; 3) PTPRG activation inhibits chemoattractant-induced LFA-1 affinity triggering and mediated adhesion in human primary monocytes; 4) PTPRG activation consistently affects the structure of chemoattractant-triggered phosphonetworks; 5) JAK2 mediates LFA-1 activation and mediated adhesion in human monocytes; and 6) JAK2 is a direct target of PTPRG tyrosine phosphatase activity, and this correlates with the antiadhesive role of PTPRG.

### Discussion

The table shows the 31 identified proteins whose tyrosine phosphorylation is affected by PTPRG activation. Values reported in % columns are percentage increase or decrease of protein tyrosine phosphorylation upon fMLF triggering in P1-WD–treated versus P1-treated monocytes. For some proteins (EGFR, INSR, KDR, ITGB1, CGR13, PTK2, PXN, SRC, and ZAP70) multiple phosphotyrosine residues are detected. From left to right, columns are HGNC protein symbols (in alphabetical order), phosphosites (p-Sites), percentage changes of phosphorylation (induced by P1-WD), and the putative functional effect, inferred from literature data mining. EGFR and PTK2 are highlighted in bold type because the functional effect could not be unambiguously inferred.

#### JAK2 is a PTPRG target

Finally, we wanted to confirm, with different techniques, that JAK2 is effectively a direct PTPRG target. First, we show in ELISA assays that in monocytes treated with the P1-WD peptide (Fig. 7A) or with TAT-ICD (Fig. 7B) and then stimulated with fMLF, JAK2 tyrosine phosphorylation is effectively reduced, thus indicating inhibition of the JAK2 tyrosine kinase activity by PTPRG activation. Second, we confirmed these data by Western blot analysis. Indeed, also in this case, the JAK-ICD and P1-WD almost completely prevented fMLF-induced tyrosine phosphorylation of JAK2, whereas the control P1, P1-WD_scrambled, and TAT peptides were completely ineffective (Fig. 7C, 7D). Finally, we performed a protein–protein interaction assay based on a substrate trapping approach by using the phosphatase-defective PTPRG D1028A mutant (31). This approach showed JAK2 direct interaction with TAT-ICD_D1028A, but not with the negative control TAT-eGFP. Interestingly, we also observed an increased JAK2 binding upon fMLF treatment, suggesting that the JAK2 tyrosine phosphorylation may increase the binding affinity for PTPRG. Altogether, these data are qualitatively and quantitatively in keeping with the high-throughput Ab array analysis and demonstrate that JAK2 is a direct PTPRG target, thus indicating that the antiadhesive effect of PTPRG involves JAK2 tyrosine dephosphorylation.

### Table I. Summary of Kinexus high-throughput phosphoproteomics data

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<th>%</th>
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The table shows the 31 identified proteins whose tyrosine phosphorylation is affected by PTPRG activation. Values reported in % columns are percentage increase or decrease of protein tyrosine phosphorylation upon fMLF triggering in P1-WD–treated versus P1-treated monocytes. For some proteins (EGFR, INSR, KDR, ITGB1, CGR13, PTK2, PXN, SRC, and ZAP70) multiple phosphotyrosine residues are detected. From left to right, columns are HGNC protein symbols (in alphabetical order), phosphosites (p-Sites), percentage changes of phosphorylation (induced by P1-WD), and the putative functional effect, inferred from literature data mining. EGFR and PTK2 are highlighted in bold type because the functional effect could not be unambiguously inferred.
Receptor homodimerization is widely recognized as the inhibitory mechanism of RPTP catalytic activity. Depending on the RPTP subtype, proposed models either suggest a constitutive homodimerization under physiological conditions, with ligand binding leading to dimer dissociation and release of the intrinsic phosphatase activity, or a ligand-induced dimerization, thus leading to phosphatase activity inhibition (5, 32). Data obtained in different cellular contexts suggest a role for a juxtamembrane WD in homodimerization (5). In the present study, we applied a CPP-based approach to investigate the function of PTPRG in primary leukocytes. The P1-WD peptide activated a tyrosine phosphatase activity only in membranes isolated from PTPRG-expressing monocytes and, accordingly, the P1-WD peptide was active only on monocyte adhesion, showing no effect in PMNs. Moreover, at the cellular level, P1-WD and TAT-ICD had the same effects on cell adhesion and on LFA-1 affinity triggering by fMLF. Finally, both P1-WD and TAT-ICD induced JAK2 tyrosine dephosphorylation on the same amino acid residues, and its effect on cell adhesion fully correlates with the effect of JAK inhibitors. Altogether, these data suggest a model in which PTPRG is constitutively expressed on monocyte plasma membrane as a homodimer with the WD involved in catalytic domain blockade. Notably,

**FIGURE 4.** Network reconstruction and modular decomposition of PTPRG-influenced protein tyrosine phosphonetwork. (A) The unique connected network component generated by the binary interactions between the 31 identified PTPRG targets. No isolated nodes are present. In blue are the 15 proteins whose signaling activity is inhibited by PTPRG activity; in red are the 14 proteins whose signaling activity is activated by PTPRG activity. The activation state of PTK2 and EGFR was not inferred. Edges between nodes are directed and indicate the direction of signaling flow within the network, thus suggesting the direction of functional influence between the identified proteins. (B and C) Topological clusters of interacting nodes obtained by means of network modular decomposition; module (B) (4.6 clustering score) and module (C) (5.5 clustering score) prevalently include activated or inhibited proteins, respectively. In blue are proteins whose signaling activity is inhibited by PTPRG activity; in red are proteins whose signaling activity is activated by PTPRG activity.
among the various RPTPs, PTPRG has the highest enzymatic activity (15), thus suggesting the importance of tight regulation by dimerization. Although previous data show a peculiar tendency of PTPRG catalytic domains to dimerize (15), in keeping with our model, structural data obtained in a cell-free system challenged the importance of the WD (15). This discrepancy could reflect differences in the experimental setting. Because our data were obtained under fully physiological conditions, we hypothesize that the juxtamembrane PTPRG WD may assume a regulatory relevance once the molecule is expressed in the lipid membrane environment. The importance of the lipid environment is also suggested by the capability of triphosphorylated nucleosides to upregulate PTPRG activity only in the context of cell membranes (33). This was also previously conjectured for other RPTPs (15).

FIGURE 5. JAK2 PTK is activated by fMLF stimulation. Monocytes were treated with buffer (Resting) or with 50 nM fMLF for the indicated times. (A) Time course of JAK2 activation by phospho-JAK2 ELISA assay; mean values of 3 experiments in duplicate are shown; errors bars are SDs. (B) Time course of JAK2 activation by Western blot analysis (representative experiment of three). (C) Western blot immuno band quantification; mean values of the three experiments are shown; the relative ratio of the band intensity of phospho-JAK2 was normalized to the level of JAK2 band intensities. (D) Effect of JAKs inhibitors on JAK2 activation. Monocytes were treated with buffer (Resting and control), 100 μM AG490, 40 μM P1 or P1-TKIP peptides, or 200 μM WHI-P154 for 1 h at 37˚C and stimulated with 50 nM fMLF for 180 s. Cell lysates were probed with anti–phospho-JAK2 and with anti-JAK2; a representative experiment of three is shown. (E) Western blot immuno band quantification; the relative ratio of the band intensity of phospho-JAK2 was normalized to the level of JAK2 band intensities; mean values of three experiments are shown. ***p < 0.001 versus Resting, CTRL, or P1. CTRL, control.

FIGURE 6. JAK2 PTKs control LFA-1 high-affinity-state triggering and mediated monocyte adhesion by fMLF. Static adhesion assays on (A) fibrinogen or (B) ICAM-1 are shown. Human monocytes were treated with buffer (control), 40 μM P1 or P1-TKIP peptides, 100 μM AG490, or 200 μM WHI-P154 for 1 h at 37˚C and stimulated with 50 nM fMLF for 60 s; mean values of six experiments in triplicates are shown; errors bars are SDs. (C) Underflow adhesion assay. Human monocytes were treated with buffer (control), 100 μM AG490, 40 μM P1 or P1-TKIP peptides, or 200 μM WHI-P154 for 1 h at 37˚C. Shown are percentages of rolling and arrested cells for the indicated times over total interacting cells; mean values of five experiments are shown; errors bars show SDs. **p < 0.01, ***p < 0.001 versus CTRL or P1. CTRL, control.
FIGURE 7. JAK2 is direct substrate of PTPRG tyrosine phosphatase activity. Phospho-JAK2 ELISA assays on human monocytes treated with (A) buffer (control), 50 µM P1-WD, or P1 or P1-WD_scrambled peptides or (B) with buffer (control), 0.5 µM TAT-ICD, or 50 µM TAT peptide for 1 h at 37˚C and stimulated with 50 nM fMLF for 90 s are shown. Shown are percentages of fold increases over the control; mean values of three experiments in triplicates are shown; errors bars are SDs. (C) Effect of TAT-ICD and P1-WD on JAK2 activation evaluated by Western blot analysis. Monocytes were treated with buffer (Resting, control), 0.5 µM TAT-ICD, 50 µM TAT peptide, 50 µM P1-WD, P1, or P1-WD_scrambled peptides for 1 h at 37˚C and stimulated with 50 nM fMLF for 180 s. Total lysates were probed with anti–phospho-JAK2 or with anti-JAK2; a representative experiment of three is shown. (D) Western blot immunoreactive band quantification; the relative ratio of the band intensity of phospho-JAK2 was normalized to the level of JAK2 band intensities; mean values of three experiments are shown. (E) JAK2 interacts with TAT-ICD_D1028A. Immobilized TAT-ICD_D1028A and TAT-eGFP were incubated with total cell lysates derived from monocytes treated with buffer or with 50 nM fMLF. JAK2 bound to the affinity resin was detected by Western blot with anti-JAK2 Ab. (F) Western blot immunoreactive band quantification; mean values of three experiments are shown; errors bars are SDs. ***p < 0.001 for resting TAT-ICD_D1028A versus resting TAT-eGFP, ***p < 0.01 for fMLF 50 nM TAT-ICD_D1028A versus fMLF 50 nM TAT-eGFP. **p < 0.01, ***p < 0.001 versus CTRL, TAT, or P1. CTRL, control.
A complex signaling network, consisting of at least 67 molecules (2), allows adaptation of circulating leukocytes to various environments, thus ensuring the completion of the migration process in response to chemoattractants. Notably, most of the studies describe proadhesive signaling events, even when antiadhesive signals likely have an equally relevant role in leukocyte recruitment, for example, during the on–off turnover of integrin activation necessary for cell motility and transmigration. To date, few signaling molecules have been shown to negatively regulate leukocyte integrin activation; these include PKA (34), RHOD (35), CDC42 (10, 36), SRC (37–39), H-RAS (40), ILK (41), FAM65B (42), and CAPN2 (43), with only CDC42 investigated according to the previously formalized four criteria (2). In the present study, fully compliant with these criteria, we show that PTPRG is a novel negative regulator of LFA-1 high-affinity-state triggering and mediated arrest by chemoattractants in human primary monocytes. Notably, PTKs of the JAK and SRC families have a regulatory role in LFA-1 affinity triggering, with JAKs showing a positive role (3), whereas SRCs possibly have a negative role (37). In our context, SRC appears inhibited by PTPRG activation (Table I), thus making it unlikely that the antiadhesive effect of PTPRG is mediated by SRC activation. Then, the most obvious possibility was that PTPRG could interfere with JAK activity. We tested this hypothesis by first showing that JAKs, as previously demonstrated in primary T lymphocytes (3), are critically involved in LFA-1 affinity triggering also in monocytes. Following this, the combined phosphoproteomics analysis showed that PTPRG activity induces JAK2 dephosphorylation on the adjacent 1007–1008 tyrosine residues. Because tyrosine 1007 and 1008 are sites of JAK2 autophosphorylation critical to kinase activity (44), this suggests that JAK2 is directly dephosphorylated and inhibited by PTPRG. This result is also in keeping with the observation that PTPRG contains a secondary substrate-binding pocket (15) capable of interacting with proteins containing adjacent phosphotyrosines, such as JAK2 upon IMLF triggering. Importantly, this was fully confirmed by the capability of the substrate-trapping PTPRG mutant (TAT-ICD D1028A) to bind with higher efficiency tyrosine phosphorylated JAK2 (Fig. 7E, 7F). Notably, previous data obtained by means of genetic deletion suggested that JAKs could be targets of CD45 (PTPRC), although not in the context of chemoattractant signaling (9). To our knowledge, our data, based on CPP technology, provide the first direct demonstration in primary leukocytes that JAKs are target of PTPRG-induced dephosphorylation, clearly indicating that the antiadhesive role of PTPRG is, at least partially, mediated by direct JAK2 blockade. Considering that PTPRG expression appears mainly restricted to monocytes and B cells, with no expression in PMNs or other leukocytes (19) (see also http://humanproteomemap.org), our data show a cell-specific mechanism of downmodulation of LFA-1 affinity triggering and mediated cell recruitment by PTPRG. Notably, although we circumvented the constitutive inhibition with the CPP approach, a relevant, still open question concerns the modality of PTPRG activation under physiological conditions, such as during monocyte adhesion in the microcirculation. The extracellular segment of PTPRG includes one carbonic anhydrase domain and one fibronectin type 3 domain. One interesting possibility is that extracellular ligands may interact with the PTPRG fibronectin type 3 domain, a motif commonly found in adhesion molecules, in turn dissociating the homodimer and thus freeing the phosphatase activity (32). It is tempting to speculate that under these conditions PTPRG, once activated, could provide a stop signal to monocyte recruitment, thus behaving as an important mechanism of immune system modulation. This interesting hypothesis will be the focus of further investigation.

The high-throughput phosphoproteomics analysis showed that PTPRG may affect the tyrosine phosphorylation of several signaling proteins. Besides JAK2, which was more deeply analyzed owing to its known role in integrin affinity triggering (3), many tyrosine kinases appear to be influenced by PTPRG activation. The combined phosphoproteomics and network topological analysis suggest a focus on SRC, KIT, PTK2, EGFR, PDGFRB, ABL1, and BTK. Among these, SRC, KIT, PDGFRB, ABL1, and BTK appear unambiguously inhibited by PTPRG. In the context of LFA-1 activation, it is reasonable to exclude SRC (37) and also PDGFRB, which do not mediate chemoattractant signaling. Taken together, these observations suggest that KIT, ABL1, and BTK may be involved in LFA-1 affinity modulation by chemoattractants, perhaps concurrently cooperating with JAKs. Interestingly, ABL1 was already shown to be a direct PTPRG target (45). A role for these kinases in LFA-1 affinity modulation is unknown, and it will be of interest to investigate their involvement in leukocyte trafficking.

In conclusion, our study describes a novel approach to investigate the regulatory role of PTPRG in primary cells, based on activation of the tyrosine phosphatase activity. By exploiting this approach, we show that PTPRG is a JAK phosphatase and negatively regulates chemoattractant signaling to LFA-1 affinity upregulation in human primary monocytes, thus suggesting a critical role for PTPRG in immune system regulation. Besides chemoattractant-triggered signaling leading to monocyte integrin activation, our study suggests that PTPRG could affect many signaling pathways also in other cellular contexts in which a dysregulated tyrosine kinase activity possibly exists, for example, in neoplastic cells (28, 46). Notably, many neoplastic cells modulate the expression of PTPRG (19). In this context, PTPRG has been recently proposed as a tumor suppressor gene involved in the pathogenesis of CML (45). Furthermore, KIT and ABL1, PTPRG targets, are oncogenes. Thus, it will be of great relevance to exploit our CPP-based approach to study the role of PTPRG in cancer development and progression.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


