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PSTPIP2, a Protein Associated with Autoinflammatory Disease, Interacts with Inhibitory Enzymes SHIP1 and Csk

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Mutations in the adaptor protein PSTPIP2 are the cause of the autoinflammatory disease chronic multifocal osteomyelitis in mice. This disease closely resembles the human disorder chronic recurrent multifocal osteomyelitis, characterized by sterile inflammation of the bones and often associated with inflammation in other organs, such as the skin. The most critical process in the disease’s development is the enhanced production of IL-1β. This excessive IL-1β is likely produced by neutrophils. In addition, the increased activity of macrophages, osteoclasts, and megakaryocytes has also been described. However, the molecular mechanism of how PSTPIP2 deficiency results in this phenotype is poorly understood. Part of the PSTPIP2 inhibitory function is mediated by protein tyrosine phosphatases from the proline-, glutamic acid-, serine- and threonine-rich (PEST) family, which are known to interact with the central part of this protein, but other regions of PSTPIP2 not required for PEST-family phosphatase binding were also shown to be indispensable for PSTPIP2 function. In this article, we show that PSTPIP2 binds the inhibitory enzymes Csk and SHIP1. The interaction with SHIP1 is of particular importance because it binds to the critical tyrosine residues at the C terminus of PSTPIP2, which is known to be crucial for its PEST-phosphatase-independent inhibitory effects in different cellular systems. We demonstrate that in neutrophils this region is important for the PSTPIP2-mediated suppression of IL-1β processing and that SHIP1 inhibition results in the enhancement of this processing. We also describe deregulated neutrophil response to multiple activators, including silica, Ab aggregates, and LPS, which is suggestive of a rather generalized hypersensitivity of these cells to various external stimulants. The Journal of Immunology, 2015, 195: 000–000.

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Abbreviations used in this article: 3-AC, 3-α-aminocholestane; ACN, acetonitrile; BM, bone marrow; BMDDM, BM-derived macrophage; FCγR, Fc receptor; PEST-PTP, proline-, glutamic acid-, serine- and threonine-rich–family PTP; PI(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; PTP, protein tyrosine phosphatase; SFK, Src-family kinase; WT, wild type.

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trisphosphate [P(3,4,5)P₃]. This results in the reduced activity of some of its downstream effectors (1). A prototypical example of such an effector is Akt (also known as PKB), a serine/threonine kinase, which is involved in the regulation of cell activation, proliferation, metabolism, and survival, and which is recruited to the plasma membrane by P(3,4,5)P₃ for further activation (2). The deletion of SHIP1 results in the increased activity of Akt in the mast cells (3) and in the increased translocation of the Akt PH domain to the plasma membrane in neutrophils, suggesting that in myeloid cells SHIP1 regulates Akt localization and activity in vivo (4). There is also ample evidence that SHIP1 negatively regulates MAPK pathways by various mechanisms, in some cases independently of SHIP1 enzymatic activity (5).

Another critical negative regulator of leukocyte signaling is the protein tyrosine kinase Csk. It is involved in the regulation of Src-family kinases (SFKs), a family of protein tyrosine kinases indispensable to the initiation of signal transduction via ITAM-bearing immunoreceptors, and with additional and important roles in signaling by cytokine, growth factor, and pattern recognition receptors, and many others (6). Csk phosphorylates an inhibitory tyrosine residue at the C terminus of SFKs. This phosphorylated tyrosine then interacts with an SH2 domain in the same molecule, resulting in an autoinhibited conformation (7–9). Csk itself is recruited to the plasma membrane and is activated by binding to the phosphorylated tyrosine motifs of transmembrane proteins such as PAG (10, 11), LIME (12), and SCIMP (13). An additional mechanism of SFK inhibition is dependent upon protein tyrosine phosphatases (PTPs) that dephosphorylate the activation loop tyrosine necessary for SFK catalytic activity. This residue can be dephosphorylated by multiple phosphatases, including the receptor tyrosine phosphatases CD45 and CD148 (14–16), PTPs of the Shp family (17), as well as PTP LYP (PTPN22, also known as PEP in mice). In T cells, LYP/PEP forms a complex with Csk.
Together, they simultaneously dephosphorylate the activation loop tyrosine and phosphorylate the inhibitory C-terminal tyrosine of SFKs, exerting a combined inhibitory effect (18, 19). LYP/PEP is a member of a small family of PTPs known as proline-, glutamic acid-, serine- and threonine-rich–family PTPs (PEST-PTPs), consisting of only three members: PEP/LYP, PTP-HSCF (PTPN18), and PTP-PEST (PTPN12) (reviewed in Ref. 20). All three family members bind Csk via proline-rich or tyrosine-containing motifs in their central region (18, 21, 22). In addition, a conserved C-terminal homology domain of PEST-PTPs binds two related proteins, PSTPIP1 and PSTPIP2 (23, 24). These are adaptor proteins involved in the control of inflammation, and their altered function results in the development of autoinflammatory diseases (25–27).

In general, these disorders are characterized by sterile inflammation and consequential tissue damage. They are mainly the result of dysregulated activity of the innate immune system with no or limited involvement of adaptive immunity (28). The autoinflammatory disease caused by PSTPIP2 mutations is, in mice, characterized by sterile inflammatory lesions in the bones and various degrees of skin and paw inflammation (26, 27, 29). The disease closely resembles the human disorder known as chronic recurrent multifocal osteomyelitis. However, human patients with genetic alterations in the PSTPIP2 gene have not been identified yet. PSTPIP2 is a member of the F-BAR family of proteins (also known as the pomb e cdc15 homology family), characterized by the presence of N-terminal F-barc domain mediating interactions with membrane phospholipids and a C-terminal tail containing various interaction motifs (30). The F-bar domain of PSTPIP2 interacts with Phl5, P2, (31), whereas its C-terminal tail binds PEST-PTPs via the interaction motif, which includes tryptophan 232 (24, 32). The C-terminal tail of PSTPIP2 contains several tyrosines of unknown function that are phosphorylated in macrophages after exposure to M-CSF (33). Two different mouse strains where point mutations in PSTPIP2 result in the autoinflammatory disorder have been established. As a result of these mutations, one of these strains, LUP0, displays approximately a 70% reduction in PSTPIP2 protein expression (27), whereas the other strain, CMO, shows a complete absence of PSTPIP2 (34). The disease is independent of T or B cells, and it was originally attributed to the enhanced activity of macrophages and osteoclasts (27, 32, 34). In addition, more recent work has suggested that neutrophils may also be a critical cell type in disease initiation (35, 36). Similar to several other autoinflammatory syndromes, the disease appears to be, at least in part, caused by the enhanced production of IL-1β (35, 37). The molecular mechanism of how PSTPIP2 prevents autoinflammatory disease development remains largely unknown. The effects of PSTPIP2 binding to PEST-family phosphatases have been tested recently in osteoclasts and megakaryocytes, where it had an effect on their differentiation. However, in these experiments, a profound effect was also seen after the mutation of C-terminal tyrosines, which rendered PSTPIP2 especially in the case of osteoclasts essentially nonfunctional without affecting the interaction with PEST-family phosphatases (32, 38). This suggested that the binding partners of these tyrosines are critical for PSTPIP2 function. However, their identity remained unknown.

In this article, we show that these phosphorylated tyrosine residues bind the lipid phosphatase SHIP1. In addition, we also show that PSTPIP2 interacts with Csk via a mechanism that is, at least in part, independent of PEST-PTPs. Our data also bring evidence for the involvement of these inhibitory enzymes in the PSTPIP2-mediated suppression of the inflammatory response.

### Materials and Methods

#### Abs

Abs to the following Ags were used in this study: FLAG (M2) and GAPDH (from Sigma-Aldrich, St. Louis, MO); phospho-Erk (T202/Y204), phospho-Akt (S473, T308), and Myc (9B11) (Cell Signaling Technology, Danvers, MA); Csk (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA); CD18 (YTS 213.1) (Serotec, Kidlington, U.K.); B220-biotin (RA3-6B2), FITC, Ly6C-PE-Cy7, (Biolegend, San Diego, CA); B202-FTTC, DX5-biotin, F4/80-biotin, F4/80-PE, and F4/80-Alexa700 (eBioscience, San Diego, CA); Thyl.2-FTTC, CD271-FTTC, anti-FITC, and anti-biotin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany); HRP-conjugated goat anti-mouse L chain–specific Ab, HRP-mouse anti-rabbit L chain–specific, purified mouse IgGwhole molecule, goat anti-mouse Fab(1), goat anti-rat Fab(1) (Jackson ImmunoResearch, West Grove, PA); Fc Block (2.4G2) (BD Biosciences, San Jose, CA). The rabbit antiserum against SHIP1, PEP, PTP-PEST, and PTP-HSCF were a gift from Dr. A. Veile (Institut de Recherches Cliniques de Montreal, University of Montreal). The Ab against human SHIP1 was from Exbio (Vestec, Czech Republic).

The mouse mAb that recognizes murine and human PSTPIP2 was generated by the immunization of mice (F1 hybrids of BalB/c × B10A) with a full-length recombinant murine PSTPIP2 produced in Escherichia coli. Splenocytes from immunized mice were fused with Sp2/0 myeloma cells and cloned by limiting dilution. Ab production was tested by ELISA and Western blotting.

To prepare mouse or human aggregated IgG, we first purified IgG from mouse serum (Sigma-Aldrich) or human Ab serum (Invitrogen, Carlsbad, CA) on protein A-Sepharose (GE Healthcare, Uppsala, Sweden), transferred them to PBS, and concentrated them to 30 mg/ml on an Amicon Ultrafuge–30K unit (Millipore, Merck, Darmstadt, Germany). The agglutination was induced by heating to 63°C for 30 min.

#### Mice

CMO mouse strain (Cg-Pstpip2cmo/J) carrying the c.293T→C mutation in the Pstpip2 gene (26, 29) on the BalB/c genetic background, resulting in a LYP/PEP change in the Pstpip2 protein, and CMO, were obtained from The Jackson Laboratory (Bar Harbor, ME). The BalB/c and F1 hybrids of BalB/c × B10A were from the animal facility of Institute of Molecular Genetics, Academy of Sciences of the Czech Republic (Prague, Czech Republic). All the experiments in this work that were conducted on animals were approved by the Animal Care and Use Committee of the Institute of Molecular Genetics and were in agreement with local legal requirements and ethical guidelines.

#### Cell lines and primary cells

All the primary cells and cell lines were cultured at 37°C with 5% CO₂ in the following media supplemented with 10% FCS and antibiotics: WEHI-231 cell line (ATCC) in RPMI 1640, HEK293FT cells (Invitrogen), Phoenix Eco cells (Origene, Rockville, MD), J774.2 (ATCC) cells, and immortalized macrophage progenitors in DMEM. IMDM was used for immortalized granulocyte progenitors and primary granulocytes.

Bone marrow (BM) was isolated from CMO and BalB/c × B10A mice sacrificed by cervical dislocation. Full BM was cultured in DMEM conditioned with 10% L929 culture supernatant containing M-CSF to differentiate BM-derived macrophages (BMDMs). Osteoclasts were differentiated in DMEM supplemented with 20 ng/ml M-CSF and 100 ng/ml RANKL (Peprotech, Rocky Hill, NJ). Murine granulocytes were isolated from BM by negative selection using B220, F4/80, DX5, c-KIT, CD3ε, and anti-CD3ε-biotinylated Abs, and anti-biotin Microbeads, on an AutoMACS magnetic cell sorter (Miltenyi Biotec), and the purity (>90%) was determined by flow cytometry. Human granulocytes were purified from buffy coats (purchased on a commercial basis from the Blood Bank of Thomayer Hospital, Prague, Czech Republic) by sedimentation in 2% Dextran T500 (Pharmacosmos, Holbaek, Denmark) followed by Ficoll centrifugation (Pharmacosmos, Holbaek, Denmark) and settling in 2% Dextran T500 (Pharmacosmos, Holbaek, Denmark). The generation of immortalized macrophage progenitors has been described previously (39); in brief, BM was centrifuged over Ficoll-Paque PLUS gradient, and mononuclear cells were directly infected with ER-Hoxb8 retrovirus by spinoculation. The cells were cultivated in a medium conditioned with 1% LUTZ culture supernatant as a source of GM-CSF and 1 µM β-estradiol (estrogen; Sigma-Aldrich). The cells could be differentiated into macrophages by withdrawal of M-CSF from the growth medium within a week. To prepare immortalized granulocyte progenitors, we used a modified version of this protocol (39). The progenitors were first enriched by the depletion of Mac-1⁺, B220⁺, and Thy1.2⁺ by mouse BM
cells and cultured in the presence of IL-3, IL-6, and SCF (supplied as culture supernatants from HEK293 cells transduced with constructs coding for respective cytokines) for 2 d. Next, they were transduced with the same ER-HoxB8 construct described earlier. The transduced cells were enriched for the GMP progenitor population by FACS (Lin-, Sca-1, c-Kit, FcyRII, CD34) and propagated in a media containing 1 mM β-estradiol and 1% SCF-containing supernatant. Granulocyte differentiation was induced by β-estradiol withdrawal and the addition of 50 ng/ml G-CSF.

DNA constructs, transfection, and transduction

The CLG construct (generated by the PCR-mediated joining of individual overlapping fragments and subcloned to MSCV-IREs-Thy1.1; Clontech, Mountain View, CA) was composed of the SH2 and SH3 domains of human Csk (aa 1–171) followed by the kinase domain from human Lck (aa 225–509) with Y505 mutated to phenylalanine and then by a Myc tag sequence and GST from Schistosoma japonicum lacking methionine one (Fig. 1A). Murine PSTPIP2 was cloned from cDNA prepared by the reverse transcriptase (RT)-PCR-AmpF/ExtF procedure using Forward (5'-AACTCAGATGCGCTTGACCTGCAGAG-3') and Reverse (5'-CTACATGAGGTCAGGACGCACATGATC-3') primers and RT-PCR reaction products were cloned into pCRII-TOPO (Invitrogen). The construct was transfected into MC3T3-E1 cells and a stable clonal cell line was selected, from which the construct was excised and subcloned to MSCV-IRES-Thy1.1 (Clontech, Palo Alto, CA). The construct was expressed in the murine B cell line WEHI-231 via retroviral infection. Because many Csk-binding proteins are substrates of SFKs, we expected that the Lck kinase domain would phosphorylate proteins bound to the Csk SH2/SH3 module of the construct, and we thus label them for relatively easy detection in the in vitro kinase assay or else after phosphorylation in vivo. This would allow for the subsequent optimization and scaling up of the procedure to identify these proteins by mass spectrometry. The Myc-GST module was intended for the tandem purification of CLG using glutathione Sepharose followed by the second round of affinity purification with the anti-Myc Ab. However, this approach was eventually abandoned because of the major technical difficulties caused, presumably, by the competition between the construct and the endogenous murine GST present in the cell lysates. Hence we eventually used this construct in only single-step Myc immunoprecipitations, without engaging its GST moiety. In this setup, we immunoprecipitated the CLG fusion protein from WEHI-231 lysates and incubated the immunoprecipitate with γ[32P]-ATP. The proteins radioactively labeled by the CLG Lck kinase domain were then subjected to SDS-PAGE and autoradiography.

Results

The initial goal of this study was the identification of novel Csk-interacting proteins. To achieve this, we designed a construct (hereafter termed CLG) consisting of the SH2 and SH3 domains of Csk and the kinase domain from the SFK Lck, followed by the Myc tag and GST (Fig. 1A). The construct was expressed in the murine B cell line WEHI-231 via retroviral infection. Because many Csk-binding proteins are substrates of SFKs, we expected that the Lck kinase domain would phosphorylate proteins bound to the Csk SH2/SH3 module of the construct, and we thus label them for relatively easy detection in the in vitro kinase assay or else after phosphorylation in vivo. This would allow for the subsequent optimization and scaling up of the procedure to identify these proteins by mass spectrometry. The Myc-GST module was intended for the tandem purification of CLG using glutathione Sepharose followed by the second round of affinity purification with the anti-Myc Ab. However, this approach was eventually abandoned because of the major technical difficulties caused, presumably, by the competition between the construct and the endogenous murine GST present in the cell lysates. Hence we eventually used this construct in only single-step Myc immunoprecipitations, without engaging its GST moiety. In this setup, we immunoprecipitated the CLG fusion protein from WEHI-231 lysates and incubated the immunoprecipitate with γ[32P]-ATP. The proteins radioactively labeled by the CLG Lck kinase domain were then subjected to SDS-PAGE and autoradiography. This procedure revealed several proteins with molecular masses of 75, 51–55, 37, and 16 kDa in the immunoprecipitates (Fig. 1B). Whereas the 75-kDa band most likely represented the auto-phosphorylated CLG construct, the identity of the other proteins remained unknown. To identify these proteins, we performed a large-scale immunoprecipitation from WEHI-231 cell lysates (from 4 × 10⁷ cells) infected with the CLG construct followed by SDS-PAGE and Coomassie blue detection. In this way, we were able to detect a protein of 37 kDa, which likely corresponded to a 37-kDa band from the in vitro kinase assay (Fig. 1C). Mass spectrometry identification revealed that this protein was PTP2P2 (Fig. 1D, Supplemental Fig. 1). Although it was known to interact with the anti-Myc Ab. However, this approach was eventually abandoned because of the major technical difficulties caused, presumably, by the competition between the construct and the endogenous murine GST present in the cell lysates. Hence we eventually used this construct in only single-step Myc immunoprecipitations, without engaging its GST moiety. In this setup, we immunoprecipitated the CLG fusion protein from WEHI-231 lysates and incubated the immunoprecipitate with γ[32P]-ATP. The proteins radioactively labeled by the CLG Lck kinase domain were then subjected to SDS-PAGE and autoradiography. This procedure revealed several proteins with molecular masses of 75, 51–55, 37, and 16 kDa in the immunoprecipitates (Fig. 1B).

For the in vitro kinase assay, cells were lysed in the lysis buffer where NaF, Na3VO4, and EDTA were omitted. The immunoprecipitations were carried out in 6-well plates precoated with 0.1 mg/ml goat anti-mouse IgG (Sigma-Aldrich) and Myc Ab (see earlier). The immunoprecipitates were incubated with a kinase buffer (25 mM HEPES pH 7.4, 5 mM MgCl2, 5 mM MnCl2, 0.1% lauryl maltoside, and 0.1 μl of [γ-32P]-ATP per well) for 20 min at room temperature. After washing, the immunoprecipitates were eluted with a 50 μl SDS-PAGE sample buffer and subjected to SDS-PAGE and autoradiography.

Mass spectrometric analysis

Protein bands were cut from the gel, then further cut into small pieces and dehydrated in a sonic bath at 60°C several times with 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (ACN). After complete destaining, proteins were reduced by 50 mM TCEP in 0.1 M 4-ethylmorpholine acetate (pH 8.1) for 5 min at 80°C and alkylated using 50 mM iodoacetamide in 0.1M 4-ethylmorpholine acetate (pH 8.1) in the dark at room temperature. Next, the gel was washed with water and ACN and was partly dried using a SpeedVac concentrator (Savant, Holbrook, NY). Finally, the gel was reconstituted with a cleavage buffer containing 0.01% 2-ME, 0.05M 4-ethylmorpholine acetate (pH 8.1), 10% ACN, and sequencing-grade trypsin (10 ng/μl; Promega). Digestion was carried out overnight at 37°C, and the resulting peptides were extracted with 30% ACN/0.1% TFA and subjected to mass spectrometric analysis. Mass spectra were acquired using the positive ion mode on a MALDI-FTMS APEX-Ultra (Bruker Daltonics, Bremen, Germany) equipped with a 9.4 T superconducting magnet and a SmartBeam laser. The acquisition mass range was 700–3500 m/z, and 512k data points were collected. The instrument was externally calibrated using the PepMix II peptide standard (Bruker Daltonics, Bremen, Germany). This results in a typical mass accuracy of 0.05 ppm. A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% ACN/0.1% TFA was used as a MALDI matrix. One microliter of the matrix solution was mixed with 1 μl of the sample on the target and the droplet was allowed to dry at ambient temperature. After the analysis, the spectra were apodized using square sine apodization with one zero fill. The interpretation of the mass spectra was done using the DataAnalysis version 3.4 and BioTools 3.2 software packages (Bruker Daltonics, Billerica, MA). The proteins were identified by peptide mass fingerprinting using the search algorithm Mascot (Matrix Science).
then immunoprecipitated this protein from the lysates of infected cells. We could readily detect endogenous Csk in FLAG immunoprecipitates from WEHI-231 cells transfected (or not) with the CLG construct and activated (or not) with an anti-IgM Ab were incubated with γ[32P]-ATP and subjected to SDS-PAGE and autoradiography. Arrows indicate the positions of major phosphorylated species. (C) Large-scale anti-Myc (CLG) immunoprecipitation from lysates of WEHI-231 transfected (or not) with the CLG construct. The immunoprecipitates were separated by SDS-PAGE followed by staining with colloidal Coomassie blue. Arrows show the positions of the CLG construct and p37. (D) Amino acid sequence of PSTPIP2. Gray bars underline the peptides identified by mass spectrometry. Functionally important sequences and residues are also labeled (F-bar domain with a black line above the sequence, important amino acid residues by numbers above the sequence).

**FIGURE 1.** Identification of PSTPIP2 as a Csk binding protein. (A) Schematic representation of the CLG construct. (B) Anti-myc (CLG) immunoprecipitates from WEHI-231 cells transfected (or not) with the CLG construct and activated (or not) with an anti-IgM Ab were incubated with γ[32P]-ATP and subjected to SDS-PAGE and autoradiography. Arrows indicate the positions of major phosphorylated species. (C) Large-scale anti-Myc (CLG) immunoprecipitation from lysates of WEHI-231 transfected (or not) with the CLG construct. The immunoprecipitates were separated by SDS-PAGE followed by staining with colloidal Coomassie blue. Arrows show the positions of the CLG construct and p37. (D) Amino acid sequence of PSTPIP2. Gray bars underline the peptides identified by mass spectrometry. Functionally important sequences and residues are also labeled (F-bar domain with a black line above the sequence, important amino acid residues by numbers above the sequence).
immunoblotting with FLAG and Myc Abs. (C) constructs with or without an inactivating mutation in the SH2 (R107K) or SH3 (W47A) domain were subjected to Myc immunoprecipitation followed by immunoblotting with FLAG and Myc Abs. (D) Empty vector-transduced cells served as a negative control. (E) FLAG immunoprecipitates from J774 cells transfected with indicated PSTPIP2-FLAG constructs and stimulated (or not) with M-CSF were subjected to immunoblotting with phosphotyrosine (P-Tyr) and PSTPIP2 Abs. Empty vector-transduced cells served as a negative control. (F) Similar immunoprecipitation as in (D) followed by immunoblotting with Csk and FLAG Abs. Only relevant parts of the blots are shown.

FIGURE 2. PSTPIP2 interaction with Csk. (A) Lysates from PSTPIP2-FLAG or vector-transduced WEHI-231 were subjected to FLAG immunoprecipitation (IP) followed by immunoblotting (WB) with indicated Abs. (B) Lysates from WEHI-231 transfected with PSTPIP2-FLAG and the Csk-Myc constructs with or without an inactivating mutation in the SH2 (R107K) or SH3 (W47A) domain were subjected to Myc immunoprecipitation followed by immunoblotting with FLAG and Myc Abs. (C) Lysates from WEHI-231 cells expressing the WT or W232A PSTPIP2-FLAG constructs were subjected to FLAG immunoprecipitation followed by immunoblotting with Abs to the indicated proteins. Empty vector-transduced cells served as a negative control. (D) FLAG immunoprecipitates from J774 cells transfected with indicated PSTPIP2-FLAG constructs and stimulated (or not) with M-CSF were subjected to immunoblotting with phosphotyrosine (P-Tyr) and PSTPIP2 Abs. Empty vector-transduced cells served as a negative control. (E) Similar immunoprecipitation as in (D) followed by immunoblotting with Csk and FLAG Abs. Only relevant parts of the blots are shown.

tail (Y3F). Y323 and Y333 have previously been reported to be responsible for the majority of PSTPIP2 tyrosine phosphorylation (24, 32), and Y329 also seemed a good candidate phosphorylation site (see Discussion for more details on the selection of these tyrosines). Next, we expressed these constructs, as well as the W232A construct, in J774 cells stimulated (or not) with M-CSF to induce the tyrosine phosphorylation of PSTPIP2. We observed an increase in the tyrosine phosphorylation of PSTPIP2 after M-CSF treatment, which was not affected by W232A mutation (Fig. 2D). In contrast, the mutation of all three C-terminal tyrosines (Y3F) almost completely abolished PSTPIP2 tyrosine phosphorylation (Fig. 2D), which is in agreement with previously published data (24, 32). In the next experiment, we analyzed the Csk binding to these mutants. For this purpose, we also included a mutation of Y249, which, of all PSTPIP2 tyrosines, most closely resembled the Csk-binding site (12). Similar to the previous experiment, in resting cells we observed Csk binding to WT PSTPIP2 (Fig. 2E). Moreover, upon M-CSF stimulation, Csk binding to PSTPIP2 substantially increased. However, none of the tyrosine mutations were able to reduce basal or M-CSF–induced Csk binding to PSTPIP2. In addition, when we analyzed the W232A mutant of PSTPIP2, which was unable to interact with PEST-family phosphatases, we found that in nonstimulated cells Csk binding was not altered by the W232A mutation. However, the increase in Csk binding after M-CSF stimulation was lost (Fig. 2E). We also tested a number of other mutations (Supplemental Table I), as well as their combinations with W232A, but we did not observe any additional effect on Csk binding (data not shown). Unfortunately, some of the candidate binding sites could not be analyzed because their mutations resulted in instability and degradation of the mutant protein that, as a result, could not be detected in the lysates of the transfected cells. Thus, we can conclude that, in J774 cells, PSTPIP2 constitutively binds Csk and this binding is independent of PEST-PTPs. Moreover, M-CSF stimulation results in enhanced binding, which is likely mediated by PEST-PTPs.

To better understand the function of PSTPIP2 in macrophages and to find relevant situations where the signaling pathways could be regulated by PSTPIP2, we analyzed its expression and phosphorylation in this cell type. To be able to follow the endogenous protein expression, we developed a mouse mAb specific for both human and mouse PSTPIP2. The Ab recognizes transfected human and endogenous PSTPIP2 (Fig. 3B), and works well in both Western blot analysis and immunoprecipitation experiments.

It has been previously published that PSTPIP2 expression in macrophages is increased after LPS treatment, suggesting that PSTPIP2 expression may be enhanced during inflammation. Thus, we treated BMDMs with LPS or various mostly proinflammatory cytokines and followed the PSTPIP2 expression. Interestingly, PSTPIP2 was strongly upregulated, not only by LPS but also by treatment with several proinflammatory cytokines, including IFN-γ, TNF-α, and GM-CSF, whereas treatment with IL-4 (which promotes their alternative M2 differentiation) did not change the PSTPIP2 expression (Fig. 3B). These data suggest that an increase in PSTPIP2 expression is a part of a proinflammatory differentiation program in macrophages.

To obtain the more complete information about the involvement of PSTPIP2 in the regulation of inflammatory processes in macrophages, we next tested several additional receptors involved in the macrophage inflammatory response for their ability to regulate the tyrosine phosphorylation of PSTPIP2. In this experiment, we stimulated J774 cells expressing FLAG-PSTPIP2 with M-CSF, an anti-Fc receptor (anti-FcR) Ab or an Ab against an integrin β2 subunit, as well as LPS, GM-CSF, and IFN-γ. In resting cells, we observed a low level of constitutive PSTPIP2 phosphorylation (Fig. 4A). Similar to the previously published data, M-CSF treatment resulted in increased phosphorylation. Importantly, PSTPIP2 tyrosine phosphorylation was also substantially increased after FcR
cross-linking with more rapid kinetics (Fig. 4A). This phosphorylation peaked at 1 min and then gradually declined (Fig. 4B). It was accompanied by increased Csk binding, which was also more sustained. The binding of the PEST phosphatases PTP-PEST and PTP-HSCF did not seem to be influenced by FcR stimulation. However, the remaining family member PTP-PEP displayed a comparatively low level of binding in the resting cells, which increased substantially after FcR stimulation (Fig. 4B). Similar phosphorylation was also observed after integrin stimulation, although in this case the involvement of the FcRs could not be excluded because full-length Abs were used to cross-link the integrins in these experiments (Fig. 4A). We did not see any changes in PSTPIP2 phosphorylation after stimulation with LPS, GM-CSF, or INF-γ (data not shown).

The C-terminal tyrosines of PSTPIP2 were identified as major phosphorylation sites in PSTPIP2, and in some experiments they also appeared to be among the functionally most important residues in the PSTPIP2 protein (32, 38). In our experiments, Csk binding did not depend on the presence of these tyrosines. Therefore, we assumed that there must be an additional, unknown binding partner that interacts with these residues, and we attempted its identification. For this purpose, we used immortalized macrophage progenitors (see Materials and Methods). These cells are much more closely related to primary cells than tumor cell lines, and thus they are better suited for the detection of the most relevant interactions. Moreover, increased number of macrophage progenitors has been observed in CMO mice, suggesting PSTPIP2 involvement in the regulation of this population (34). Finally, these cells can be propagated in a cell culture and obtained in sufficient numbers for large-scale experiments. We infected these cells with WT PSTPIP2 and PSTPIP2 with mutated C-terminal tyrosines (Y3F) and then performed large-scale PSTPIP2 immunoprecipitations. Mass-spectrometry analysis of the proteins specifically present in the immunoprecipitates of WT, but not mutant, PSTPIP2 resulted in the identification of the lipid phosphatase SHIP1 as a novel PSTPIP2 binding partner (Fig. 5A, Supplemental Fig. 2). To confirm these data under more physiological conditions, we immunoprecipitated PSTPIP2 from the lysates of WT and CMO BMDMs stimulated with M-CSF or via FcR, and we observed a low level of constitutive SHIP1 binding that was substantially increased after both M-CSF and FcR stimulation (Fig. 5B, 5C). To verify that SHIP1 binds to the C-terminal tyrosines of PSTPIP2, we expressed WT and Y3F mutants of PSTPIP2 in CMO BMDMs and stimulated these cells with pervanadate. Indeed, we could readily observe SHIP1 binding to WT, but not to the mutant protein, confirming that functionally important C-terminal tyrosines are required for SHIP1 binding (Fig. 5D).

SHIP1 dephosphorylates the PI3K product PI(3,4,5)P₃, thus regulating a number of important pathways, whereas Csk inhibits SFKs, which are also expected to have a strong impact on macrophage signaling. Unexpectedly, we could not find any differences in the activity of M-CSF, LPS, or FcR-driven signaling pathways when comparing the BMDMs from WT and CMO mice (data not shown). This included the analysis of Akt and Erk phosphorylation, which are known to be regulated by SHIP as well as SFK phosphorylation at the inhibitory tyrosine (or in the activation loop) regulated by Csk. We also analyzed a number of other pathways in the macrophages (Jak-Stat, NF-κB, JNK, p38) (data not shown), but we did not see any defects. However, recently published work has suggested neutrophil granulocytes and IL-1β as probable driving forces behind inflammatory disease in CMO mice (35, 36). Thus, as a next step, we analyzed PSTPIP2 expression and function in granulocytes. Surprisingly, we found that
in these cells the expression of PSTPIP2 is higher than in macrophages or osteoclasts (Fig. 6A). Moreover, in contrast with our observations in the macrophages, CMO neutrophils exhibited increased FcR-triggered phosphorylation of the Erk and p38 MAPKs, as well as higher phosphorylation of Akt (Fig. 6B) and more efficient processing of IL-1β (Fig. 6C), compared with WT

**FIGURE 5.** Binding of SHIP1 to PSTPIP2. (A) Amino acid sequence of SHIP1. Gray bars underline the peptides identified by mass spectrometry. (B and C) Lysates from WT or CMO BMDMs activated for indicated time intervals with M-CSF (B) or by FcR cross-linking (C) were subjected to PSTPIP2 immunoprecipitation followed by SHIP1 or PSTPIP2 immunoblotting. (D) CMO macrophages differentiated from immortalized macrophage progenitors were transduced with the indicated constructs. The cells were treated (or not) with 0.1 mM pervanadate and the lysates from these cells were subjected to PSTPIP2 immunoprecipitation followed by SHIP1 and PSTPIP2 immunoblotting. Only relevant parts of the blots are shown.

**FIGURE 6.** Effects of PSTPIP2 deficiency on neutrophil signaling. (A) Expression of the PSTPIP2 protein in the lysates of BMDMs and BM-derived osteoclasts and primary neutrophils purified from the same organ. (B) Erk, p38, and Akt (S473) phosphorylation in the lysates of neutrophils purified from mouse BM and treated for the indicated time intervals with mouse aggregated IgG to stimulate their FcRs. (C) Neutrophils were purified from mouse BM primed for 3 h with 100 ng/ml LPS and then for the indicated time intervals with aggregated mouse IgG. They were then lysed and subjected to immunoblotting with the IL-1β Ab to probe for processed IL-1β and its precursor. Note the presence of the Ig L chains from the mouse IgG used in the FcR activation. (D) Akt phosphorylation at S473 in the lysates of neutrophils treated for 90 min with 50 ng/ml LPS. (E) Erk phosphorylation in the lysates of neutrophils treated for 15 min with silica particles (50 μg/cm²). (F) The same samples from (E) were probed for pro–IL-1β and its cleavage products. Only relevant parts of the blots are shown.
neutrophils. The additional analysis of signaling pathways in granulocytes isolated from CMO mice revealed that Akt phosphorylation was also increased after the relatively long (90-min) treatment of neutrophils with LPS (Fig. 6D). However, pro–IL-1β production after LPS priming did not appear to be altered in CMO neutrophils (Fig. 6C).

Finally, we also tested the effects of silica particles, a typical inflammasome activator, on CMO neutrophil signaling and IL-1β cleavage. The silica exposure in these cells resulted in the enhanced activation of MAPKs Erk1/2 (Fig. 6E), whereas no reproducible differences in Akt phosphorylation were observed in CMO mice (data not shown). To analyze IL-1β processing, we induced pro–IL-1β production in neutrophils by priming with LPS and then triggered its cleavage by subsequent incubation with silica. Although the LPS-induced pro–IL-1β production was not markedly affected in CMO neutrophils, silica exposure led to the enhanced production of mature IL-1β of 21 and 17 kDa (Fig. 6F), which are the sizes characteristic for cleavage by neutrophil serine proteases and inflammasome-dependent caspases, respectively (41). This is in agreement with the previously published data showing the enhanced secretion of IL-1β by neutrophils into the culture media under these circumstances (35, 36).

Because recent reports showed that IL-1β and inflammasome-activated caspases are critical for the disease development in CMO mice (35–37), we decided to analyze the silica-triggered IL-1β production in more detail, especially with respect to the interactions among PSTPIP2, SHIP1, and Csk described earlier. To address the question of PSTPIP2 phosphorylation under these conditions, we performed the immunoprecipitation of endogenous PSTPIP2 from unstimulated or LPS-primed and silica-stimulated conditions, we performed the immunoprecipitation of endogenous PSTPIP2; data not shown). Because vector-infected cells were already progressing to apoptosis at the time when the other cell lines just reached the maturity, they could not be used as a control.

Next, to directly analyze the role of SHIP1 in IL-1β processing, we treated the WT and CMO primary granulocytes from the BM

![FIGURE 7. Role of SHIP1 in the PSTPIP2-mediated suppression of IL-1β processing. (A) BM cells were primed with 50 ng/ml LPS for 3 h followed by 15-min silica exposure (50 μg/cm²). PSTPIP2 was immunoprecipitated from the lysates of these cells and the immunoprecipitates were subjected to immunoblotting with phosphotyrosine (P-Tyr) and PSTPIP2 Abs. Only relevant parts of the blots are shown. (B) Granulocytes differentiated from immortalized progenitors transduced with WT PSTPIP2 and its Y3F and W232A mutants were primed for 3 h with 100 ng/ml LPS and then stimulated by silica particles (50 μg/cm²) for the indicated time intervals. The cells were then lysed and probed for IL-1β cleavage products. (C) Neutrophils purified from the mouse BM were primed with 100 ng/ml LPS for 3 h and then with silica particles (50 μg/cm²) for the indicated time intervals in the presence or absence of the SHIP1 inhibitor 3AC (10 μM). Only relevant parts of the blots are shown.

PSTPIP2 BINDS SHIP1 AND Csk
with the specific SHIP1 inhibitor 3AC. In agreement with the proposed role of SHIP1 in PSTPIP2 function, this treatment resulted in enhanced IL-1β cleavage after exposure to silica in WT neutrophils, whereas only a very mild effect was observed in their CMO counterparts (Fig. 7C). Altogether, these data support the conclusion that interaction with SHIP1 is a part of the mechanism of how PSTPIP2 suppresses the IL-1β–mediated inflammatory response in granulocytes.

Finally, we wanted to test the expression and interactions of PSTPIP2 in primary human granulocytes. Because these cells can be obtained in relatively large numbers, they also allowed us to test the PSTPIP2 interactions. We isolated human granulocytes from fresh buffy coats and stimulated these cells with silica or human aggregated IgG (as an FcR ligand). Surprisingly, these granulocytes did not appear to interact with silica particles or to phagocytose these particles, and this treatment did not result in any PSTPIP2 tyrosine phosphorylation (data not shown). However, they vigorously responded to the FcR stimulation. This treatment resulted in the increased tyrosine phosphorylation of PSTPIP2 accompanied by the binding of Csk and SHIP1 (Fig. 8). The 100-kDa form of SHIP1 prevailing in these immunoprecipitates is likely the result of proteolytic cleavage and has been described previously (42).

These results confirm that PSTPIP2 is also expressed in human granulocytes and that it participates in similar interactions as its murine counterpart.

Discussion

PSTPIP2 is known for its principal role in the development of the inflammatory disorder described as chronic multifocal osteomyelitis in mice, where its reduced expression or its complete absence is the main cause of the disease (26, 27, 34). However, the mechanism for how PSTPIP2 prevents the disease development has remained unclear. As an adaptot protein, it is likely to function via the recruitment of inhibitory enzymes and other negative regulators. The only molecules of this class previously known to bind to PSTPIP2 were the PEST-PTPs (24, 32). All three family members have been expected to bind to PSTPIP2, although the interaction has formally been proved only for PTP-HSCF and PTP-PEST (24, 32). We filled this gap by showing that PEP/LYP also interacts with PSTPIP2 in a similar manner, dependent on the residue W232 of PSTPIP2 (Fig. 2C). Mice deficient in PEP or PTP-PEST do not develop typical signs of autoinflammatory disorder (43–46), and thus it seems that the major functions of these PTPs are not related to their binding to PSTPIP2. However, if there is a redundancy among the PEST-PTPs that are associated with PSTPIP2, single family member deficiencies would probably not reveal any relevant phenotype. Moreover, additional evidence supports the importance of PEST-PTPs for the functionality of PSTPIP2. The W232A mutation in PSTPIP2, which prevented its binding to PEST-family phosphatases, diminished the ability of PSTPIP2 to inhibit the differentiation of osteoclasts and megakaryocytes (32, 38). In addition, we show in this article that this tryptophan is also important for the suppression of IL-1β processing in neutrophils. The substrate of PSTPIP2-associated PEST-PTPs remains unknown. By analogy with PSTPIP1 (47), one might speculate that their substrate would be PSTPIP2 itself, but we have not observed any increase in the phosphorylation of PSTPIP2 mutated at W232 (Fig. 2D). Thus, it seems more likely that these phosphatases dephosphorylate some of its binding partners, such as SHIP1 and Lyn or other unknown proteins in the vicinity of PSTPIP2.

Another possible role of PEST-PTPs includes the recruitment of Csk to PSTPIP2, which is further enhanced by the independent binding of Csk directly to PSTPIP2. In such a case, SFKs would be the most probable target. We did not observe any effect of PSTPIP2 deficiency on the global phosphorylation of SFKs, whether in macrophages or granulocytes. However, it is possible that PEST-PTPs together with Csk specifically inhibit the pool of SFKs associated with PSTPIP2. The interaction between PSTPIP2 and SFK Lyn was described previously in megakaryocytes (38), and we detected a similar interaction in macrophages (data not shown). The effect of Csk binding could not be fully analyzed because we were unable to identify its binding site in PSTPIP2. We suspect that the binding site is within the F-bar domain or other sequences that could not be evaluated because of the lack of mutated protein expression. Notably, the sequence integrity seems to be essential for PSTPIP2 protein stability, because other mutations, including Lupo and CMO, also result in defective protein expression (27, 34).

In addition, there was a fraction of Csk that, in J774 cells, interacted with PSTPIP2 inducibly after M-CSF or FcR stimulation. The binding of the inducible Csk fraction was dependent on the interaction of PSTPIP2 with PEST-family phosphatases. The most likely candidate for bringing this additional Csk to the complex is PTP-PEP, which interacted with PSTPIP2 in an inducible manner. However, another option is that PTP-HSCF, which, unlike PTP-PEST or PTP-PEP, binds Csk via phosphorylated tyrosines in its C-terminal tail (22), is responsible for this inducible binding.

Another critical region in PSTPIP2 comprises the C-terminal tyrosines. Their mutation completely abolished the ability of PSTPIP2 to suppress the differentiation of osteoclasts and reduced its ability to inhibit megakaryocyte differentiation (32, 38). The most likely explanation was that these tyrosines recruit a negative regulatory molecule responsible for these effects. In this work, we have shown that these tyrosines are responsible for binding to the lipid phosphatase SHIP1. Indeed, Y329 and Y332 very well match the consensus binding site for the SHIP1 SH2 domain (48, 49), whereas Y333 bears a certain resemblance to the ITIM motif [hydrophobic amino acid (L) in position —2 with respect to the tyrosine], which is also known to bind the SHIP1 SH2 domain (50). All the previous work on PSTPIP2 tyrosine phosphorylation has analyzed only Y323 and Y333. It was based on the assumption that these tyrosines are analogous to the phosphorylated tyrosine residues found in the related and more thoroughly studied protein PSTPIP1 (Y344 and Y367, respectively) (24). However, our sequence analysis did not reveal any relationship between these
tyrosines. Y367 of PSTPIP1 is located inside its SH3 domain, which is not present in PSTPIP2. Moreover, it is a highly conserved residue forming a part of the SH3 domain ligand-binding pocket that is found within a number of these domains in various proteins where it is thought to regulate their interactions with respective ligands (51). The sequence surrounding Y344 of PSTPIP1 (LVYAS1) is also somewhat different from Y323 of PSTPIP2 (PDYSVV), and therefore we concluded that the C-terminal tyrosines are a unique feature of PSTPIP2 not found in PSTPIP1. Notably, in the previous work, the mutation of Y323 and 333 severely reduced, but did not completely abolish, the phosphorylation of PSTPIP2, suggesting that there is an additional tyrosine phosphorylation site in PSTPIP2 (24). Y329 was shown to be phosphorylated in mast cells (52), and thus we assumed that Y329 is the most likely candidate. Therefore, we decided to mutate all three C-terminal tyrosines in PSTPIP2 at the same time for our analysis of SHIP1 binding and function. This analysis showed that these three tyrosines are required for SHIP1 binding and that the PSTPIP2 phosphorylation in their absence is negligible. Moreover, we also showed that these tyrosines are required for the maximum inhibition of IL-1β processing in neutrophils. This, together with our data demonstrating increased IL-1β cleavage in WT neutrophils in the presence of the SHIP1 inhibitor, strongly supports the idea that some of the key functions of PSTPIP2 are, at least in part, mediated by SHIP1. Additional support for this idea is also derived from the fact that the Akt and Erk pathways, which are both known to be regulated by SHIP1, are affected by PSTPIP2 absence.

When considering the role of SHIP1 in PSTPIP2 function, it is also interesting to compare the phenotypes of SHIP1-deficient mice with the phenotype caused by PSTPIP2 deficiency. Similar to CMO, SHIP1−/− mice develop a disease with an autoinflammatory component where inflammatory myeloid infiltrate into multiple organs and enhanced serum IL-6 levels can be observed (3, 53–55). Both strains display splenomegaly with extramedullary hematopoiesis and an increased number and proliferation of myeloid progenitor cells (3, 34, 53). In addition, they also share a phenotype of increased osteoclastogenesis and osteoclast activity, resulting in osteopenia and osteoporosis (32, 56). These features shared between SHIP1−/− and CMO mice suggest that they are components of a common signaling pathway. In contrast, the reduced survival of SHIP−/− mice because of massive myeloid cell infiltration in the lungs is not observed in CMO mice (53), and PSTPIP2 does not seem to be involved in endotoxin tolerance. This phenomenon is mainly caused by the dramatic increase in SHIP1 expression after LPS treatment (57). Despite the fact that the expression of PSTPIP2 is also increased, we did not observe any significant difference in the signaling of endotoxin-tolerized WT and CMO BMDMs, suggesting that PSTPIP2 plays no role in this process (data not shown). The more serious phenotype of SHIP1−/− mice is, however, not surprising, because PSTPIP2 is not the only binding partner of SHIP1, and this enzyme interacts with multiple other proteins and has an effect on other pathways.

The initial characterization of CMO and Lupo mice led to the conclusion that macrophages and osteoclasts are the major cell types responsible for the disease development (27, 32, 34). However, recent work has suggested that, in fact, granulocytes are the most important cell type triggering the disease via the increased production of IL-1β (35, 36). Indeed, we had difficulty detecting any differences in the signaling pathways between the WT and CMO macrophages. In contrast, in CMO neutrophils, we observed enhanced activity of multiple signaling pathways after LPS or silica exposure or after FcR stimulation. The CMO disease development was recently shown to require the activity of inflammasome-associated caspases (36). Indeed, we have observed increased CMO neutrophil responses to a typical inflammasome stimulator silica, which was also used in the previous studies (35, 36). Moreover, we also observed enhanced responses to FcR cross-linking, and to some extent also to TLR stimulation by LPS. This suggests that PSTPIP2 has relatively broad effects on neutrophil sensitivity to various stimuli, and that it is likely that other receptors and pathways that have not been tested yet are influenced by the absence of PSTPIP2.

Which of the receptors regulated by PSTPIP2 are responsible for disease initiation in vivo remains unclear. One possibility is that a receptor for environmental silica or other related compounds triggers the disease in living animals. Our data, as well as previous reports, show an exaggerated neutrophil response to silica stimulation. However, to our knowledge, the silica receptor on granulocytes has not been identified yet, and so how silica particles initiate phagocytosis and signaling remains unclear. Fc receptors are also good candidates for triggering the disease, because they not only recognize Abs but they also bind pentraxins and pentraxin-opsonized particles (58). Pentraxins, such as C-reactive protein and serum amyloid P, are a family of acute-phase proteins involved in the recognition and opsonization of bacteria, and could well represent a link described by Lukens et al. (36) between altered bacterial microflora in the gut and inflammatory disease development in CMO mice. Moreover, FcRs use a relatively universal ITAM-dependent signaling pathway, which is also used by a number of other innate immune receptors (e.g., the C-type lectin family of pattern recognition receptors and the TREM family receptors), as well as integrins (59), and our results thus imply that signaling by these receptors will also potentially be affected. Clearly, additional research is needed to address all these possibilities, but at present our data suggest that pathways downstream of multiple receptors are affected by PSTPIP2 deficiency and, in fact, they all may contribute in concert to the CMO disease development. Our data provide evidence that SHIP1 and Csk are part of the PSTPIP2-dependent inhibitory network that regulates these pathways and prevents the development of autoinflammatory disease. Deciphering the mechanisms that govern the assembly and activity of this network will help us to understand the cause of autoinflammatory diseases, including chronic recurrent multifocal osteomyelitis and other related disorders, and will facilitate the development of successful treatments.

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Disclosures

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