This information is current as of April 14, 2017.

PC3-Secreted Microprotein Is a Novel Chemoattractant Protein and Functions as a High-Affinity Ligand for CC Chemokine Receptor 2

Xiaolei Pei, Qianying Sun, Yan Zhang, Pingzhang Wang, Xinjian Peng, Changyuan Guo, Enquan Xu, Yi Zheng, Xiaoning Mo, Jing Ma, Dixin Chen, Yang Zhang, Yingmei Zhang, Quansheng Song, Shuai Guo, Taiping Shi, Zhixin Zhang, Dalong Ma and Ying Wang

J Immunol published online 17 January 2014
http://www.jimmunol.org/content/early/2014/01/17/jimmunol.1300758

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
PC3-Secreted Microprotein Is a Novel Chemoattractant Protein and Functions as a High-Affinity Ligand for CC Chemokine Receptor 2

Xiaolei Pei,*1 Qianying Sun,*1 Yan Zhang,*1 Pingzhang Wang,*1,†,‡ Xinjian Peng,* Changyuan Guo,* Enquan Xu,* Yi Zheng,* Xiaoning Mo,* Jing Ma,* Dixin Chen,* Yang Zhang,* Yingmei Zhang,*1 Quansheng Song,*† Shuai Guo,‡ Taiping Shi,‡ Zhixin Zhang,§ Dalong Ma,*1,‡ and Ying Wang*1,†

PC3-secreted microprotein (PSMP) or microseminoprotein is a newly discovered secreted protein whose function is currently unknown. In this study, PSMP was found to possess chemoattractant ability toward monocytes and lymphocytes, and its functional receptor was identified as CCR2B. PSMP was identified as a chemoattractant protein from a PBMC chemoattractant platform screen that we established. The mature secreted PSMP was able to chemoattract human peripheral blood monocytes, PBLs, and CCR2B-expressing THP-1 cells, but not peripheral blood neutrophils, even though it does not contain the classical structure of chemokines. CCR2B was identified as one receptor for PSMP-mediated chemotaxis by screening HEK293 cells that transiently expressed classical chemokine receptors; results obtained from the chemotaxis, calcium flux, receptor internalization, and radioligand-binding assays all confirmed this finding. To further identify the major function of PSMP, we analyzed its expression profile in tissues. PSMP is highly expressed in benign prostatic hyperplasia and in some prostate cancers, and can also be detected in breast tumor tissue. In response to PSMP stimulation, phosphorylated ERK levels downstream of CCR2B signaling were upregulated in the PC3 cell line. Taken together, our data collectively suggest that PSMP is a chemoattractant protein acting as a novel CCR2 ligand that may influence inflammation and cancer development. The Journal of Immunology, 2014, 192: 000–000.

Chemokine receptors are a family of structurally related proteins (1). Chemokines are divided into four major subfamilies, CC, CXC, CX3C, and XC, based on the arrangement of their first two N-terminal cysteine residues. The first two cysteine residues can be adjacent to each other (CC), contain a single amino acid (CX3C) between them, contain three random amino acids (CXC) between them, or lack the first cysteine residue (XC). Chemokines can mediate their activities through G-protein–coupled receptors having a characteristic seven-transmembrane structure and transduce their signals to the inside of the cell through heterotrimeric G-proteins (2). The chemokine superfamily plays an important role in acute and chronic inflammation (3, 4), cancer development (5, 6), and cardiovascular disease (7, 8).

Members of the “chemokine-like function” (CLF) group cannot be classified into known chemokine subfamilies but do share some structural or functional features with classical chemokines and can signal through chemokine receptors. Several CLF chemokines that bind and activate chemokine receptors have been identified, such as MIF (9), β-defensins (10), and a tyrosyl-tRNA-synthetase fragment (11), among others.

Our laboratory focuses on finding new human gene encoding proteins that have potential chemokine or CLF. After the completion of the Human Genome Project, we established a database containing potential cytokines that includes 212 candidate genes, none of which have any currently reported function, immune related or otherwise. We used a PBMC chemoattractant platform to identify candidate chemoattractant cytokines and found that PC3-secreted microprotein (PSMP), or microseminoprotein (MSMP) (12), a gene whose function is currently unknown, exhibited chemotaxis toward PBMCs.

PSMP, or MSMP, homologous to β-microseminoprotein, is predicted to be a secreted protein containing a signal peptide sequence without the classical structure of chemokines. This protein is strongly and with relative specificity expressed in the hormone-insensitive PC3 prostate cancer cell line and is also expressed in human prostate cancer (12). However, its function remains unknown. In this article, we demonstrate that mature, secreted PSMP could induce migration of human peripheral blood monocytes (PBMs), PBLs, and the human monocytic leukemia THP-1 cell line. The chemoattractant receptor for PSMP was determined to be CCR2B through functionally screening the classical chemokine receptors. Furthermore, PSMP may function in inflammation and cancer development, as PSMP was highly expressed in both benign...
prostatic hyperplasia (BPH) and some prostate cancers, and could be detected in breast tumor tissues.

Materials and Methods

Materials, reagents, and chemicals

RPMI 1640 medium and FBS were purchased from Life Technologies, DME broth (BFA), BSA, and mouse anti-CD21/32 were purchased from Sigma-Aldrich. Pertussis toxin (PTX) was purchased from Alexis Biochemical. 125I Na was obtained from DuPont. CCLX, CCL12, CCL2, CCL5, and CCL8 were purchased from PeproTech. Fluo-3 AM was obtained from Invitrogen. PE goat anti-mouse IgG was purchased from Bio-Rad. Pertussis toxin (PTX) was purchased from Alexis Biochemical. Flow cytometry reagents (BD Biosciences, San Jose, CA) were used to analyze the CCR2B receptor expression on HEK293 cells. PE goat anti-mouse IgG was purchased from Bio-Rad Laboratories, and ECL plus was purchased from GE Healthcare.

Expression and purification of recombinant proteins

The primers were designed using the sequence from the following GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) submission of PSMP: NM_001044264.2. The PSMP-myc-his DNA sequence was integrated into the pcDB plasmid. The pcDB-PSMP plasmid was transfected with poly-ethyleneimine (Sigma-Aldrich) into the HEK293 cells (ATCC) by electroporation at 120 V for 30 ms, using an electric pulse generator (Electro Square Porator ECM 830; BTX, San Diego, CA). After 12 h post transfection with Hektor S medium (Cell Culture Technologies, Zurich, Switzerland) with 2% glutamate (Sigma-Aldrich), media containing mature secreted PSMP were collected on the sixth day after media replacement. Recombinant PSMP was purified using Ni Sepharose High Performance according to the manufacturer’s instructions (GE). Finally, PSMP protein was eluted by 500 mM imidazole (pH 8.0), and imidazole was replaced with PBS (pH 7.2) by 3000 m.w. Millipore. All glassware used to purify PSMP was pretreated at 180°C for 4 h to eliminate endotoxin. The recombinant PSMP was dissolved in PBS (pH 7.2) at 600 ng/µl and stored in −80°C.

The preparation of rabbit anti-PSMP polyclonal Ab and mouse anti-PSMP mAbs

The PSMP prokaryotic protein was used to immunize the rabbit and mice. The polyclonal Ab was purified from the rabbit’s serum by PSMP peptide coupling Sepharose 4B. After the mice were immunized, the B cells in the spleen were acquired and fused with the hybridoma cell line. The positive clones were selected, the supernatant of which contained the mAb anti-PSMP. The mAbs were purified by protein-G.

N-terminal sequencing

The purified secreted PSMP protein was separated by SDS-PAGE electrophoresis and transferred onto a hydrophobic polyvinylidene fluoride membrane (Whatman), which was then stained in Coomassie blue staining solution. The stained PSMP band was sequenced by Shanghai GeneCore Biotechnologies.

Human peripheral blood cell separation and chemotaxis assay

Human peripheral blood was obtained from the Beijing Red Cross Blood Center. Peripheral leukocytes were separated by Polymorphrep (Axis-Shield, Oslo, Norway) at a 1:077 density following the manufacturer’s instructions. PBMCs were located at the interface between RPMI 1640 and Polymorphrep and were counted in our laboratory. PBMCs were located at the interface between Polymorphrep and RBCs. After washing PMNs twice with RPMI 1640, a Boyden chamber assay (Neuro Probe) was carried out with RPMI 1640, a Boyden chamber assay (Neuro Probe) was carried out. HEK293 cells were loaded with fluo-3 AM, washed twice with PBS, and stimulated by 100 nM CCL2, 100 nM CCL8, or 200 nM PSMP. Calcium flux was measured using a Leica SP2 confocal laser-scanning microscope; images were acquired every 5 s, and the first 35 s were used as the basal calcium flux line before any stimulation was added. Calcium flux was recorded for 3 min before the second stimulation. Calcium flux intensity was assessed by Leica System Analysis Software.

Receptor internalization assay

CCR2B was expressed in HEK293, as described above. HEK293-CCR2B was transfected with CCL2, PSMP, or BSA in suspension for 1 h. Cells were then stained with anti-CCR2 or mouse IgG primary Abs followed by goat anti-mouse-PE IgG as the detection Ab. CCR2 expression was then analyzed on a FACS Calibur flow cytometer (Becton Dickinson).

In the receptor internalization assay using confocal microscopy, the CCR2B-EGFP plasmid was transiently expressed in HEK293 as described above. The transfected cells were stimulated with 0.1 and 1 µM PSMP or CCL2 or BSA for 1 h in supernatant, and the subcellular localization of CCR2-EGFP was observed under a confocal microscope (Leica TCS SP8).

Radioligand binding assay

An HEK293 cell line stably expressing CCR2B-EGFP was established. PSMP was labeled with [125I]. For saturation experiments, equivalent quantities of [125I]-PSMP were incubated with varying quantities of CCR2B cell membrane extract in our laboratory. The percentage of binding was expressed as the number of bound radioligand minus the number of bound radioligand in the presence of an excess of unlabeled PSMP. Clouds containing radioactivity were evaluated. Results were corrected for “blank” background radiation, defined as the radioactivity precipitated by polyethylene glycol–γ-globulin from the same amount of radioactive ligand in binding buffer in the absence of cells.

Reverse transcription and real-time quantitative PCR

Total RNA was purified from cultured cells using the RNeasy Mini Kit (Invitrogen) following the manufacturer’s protocol. After the reverse transcription step (50°C for 50 min), 28 PCR cycles amplified the PSMP product, and PCR products were analyzed on 1% agarose gel. The primers for amplification were as follows: PSMP, 5'-CCAGGACTCTACAGCA-GTGTACAC-3' (forward), 5'-ACGAGCAGGACCAGGCTGAC-3' (reverse); GAPDH, 5'-ACCAGGATCATGCATAC-3' (forward) and 5'-TCCACCCACCTGTGCT-3' (reverse). Tissue libraries were purchased from Clontech Laboratories. Real-time PCR was performed in the ABI 7300 System (Applied Biosystems, Foster City, CA) using the human Universal Probe Library system (Roche) and Taqman Gene Expression Master Mix (Applied Biosystems). Cycling conditions were as follows: 10 min at 95°C for the denaturing step, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Samples were run in triplicate. All samples were normalized against GAPDH using the comparative CT method. The primer sequences used were as follows: PSMP, 5'-CTCTTGTC-TGACGTGCCCTGG-3' (forward), 5'-TCCCTGCCCTGGACTAC-3' (reverse); GAPDH, 5'-TCCTGACTGCTTCCAC-3' (forward), 5'-GGCAGGATGACACCTTT-3' (reverse). Universal Probe Library probes no. 66 for PSMP and no. 45 for GAPDH were used.
**Immunohistochemistry**

Tissue paraffin sections were pretreated in 65°C for ≥ 4 h. The sections were hydrated in 100%, 95%, 90%, 80%, 75%, and 70% ethanol. Ag was retrieved by the high-pressure method, and peroxidase was removed by 3% H2O2 for 15 min. After sections were blocked by goat serum in working buffer for 30 min, 1 μg/ml rabbit anti-PSMP Ab or IgG was applied onto the section for 1 h at 37°C. The tissue section was washed three times in PBS (pH 7.2), and the Diaminobenzidine Detection System (DAKO code k5007) was used to visualize expression. PSMP expression was scored according to the intensity of immunohistochemical staining, using the following scale: 0 = no staining; 1 = weak staining; 2 = distinct staining; 3 = very strong staining.

**ELISA**

PSMP secreted into the culture medium of PC3, DU145, MCF7, and MDA-MB-231 cell lines (ATCC) was evaluated by ELISA using anti-PSMP Abs. Rabbit polyclonal anti-PSMP was used as the capture Ab, and mouse monoclonal anti-PSMP, 3D5, was used as the detection Ab. The capture Ab was coated overnight in coating buffer. After blocking in 5% BSA for 30 min, cell supernatant was added and incubated with the capture Ab for 2 h. The supernatant was discarded, and samples in wells were washed five times with PBS containing 0.05% Tween 20 (PBST). The detection Ab was then added for 2 h. After another five washes with PBST, the HRP-conjugated rabbit anti-mouse IgG (H + L) was added as the secondary Ab for 30 min. After six washes with PBST, the samples were developed using the diaminobenzidine system.

**Western blot**

Cell lysates were extracted with RIPA lysis buffer and cleared by centrifugation at 16,000 × g for 15 min at 4°C. Total protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences). After blocking the nitrocellulose membranes in ODYSSEY block buffer for 1 h, membranes were incubated with primary Abs at 4°C overnight and then with IRDye 680/800–labeled IgG secondary Ab at room temperature for 1 h. Finally, fluorescence intensity was detected on the membranes with the LI-COR Infrared Imaging System and analyzed using ODYSSEY software.

**Statistical analysis**

Statistical analysis was performed using the two-tailed Student t test (unpaired) for determining differences between means in the chemotaxis, calcium flux, and tissue microarray assays. Data were expressed as mean ± SEM. The significant differences between groups are represented by *0.01 < p < 0.05, **0.001 < p < 0.01, and ***p < 0.001. For the chemotaxis assay, every chemoattractant protein was tested in triplicate wells, and the significant differences in chemoattractant activity between two proteins were evaluated in Prism 5.0 (GraphPad Software). In the calcium flux assay, several cells (n > 8) were randomly selected in each image, and the optical cell density in a series of images from 0 to 350 s was evaluated by the Leica System Analysis Software. The calcium-flux change curve generated from the cells in one image over time was analyzed in Prism 5.0 (GraphPad Software).

**Results**

**The expression, purification, and analysis of recombinant human PSMP**

The pcDB-PSMP plasmid was transiently transfected into the HEK293T cell line using the polyethyleneimine system. The mature secreted PSMP-myc-his recombinant protein was successfully secreted into the culture medium of PC3, DU145, MCF7, and MDA-MB-231 cell lines (ATCC) was evaluated by ELISA using anti-PSMP Abs. Rabbit polyclonal anti-PSMP was used as the capture Ab, and mouse monoclonal anti-PSMP, 3D5, was used as the detection Ab. The capture Ab was coated overnight in coating buffer. After blocking in 5% BSA for 30 min, cell supernatant was added and incubated with the capture Ab for 2 h. The supernatant was discarded, and samples in wells were washed five times with PBS containing 0.05% Tween 20 (PBST). The detection Ab was then added for 2 h. After another five washes with PBST, the HRP-conjugated rabbit anti-mouse IgG (H + L) was added as the secondary Ab for 30 min. After six washes with PBST, the samples were developed using the diaminobenzidine system.

**Western blot**

Cell lysates were extracted with RIPA lysis buffer and cleared by centrifugation at 16,000 × g for 15 min at 4°C. Total protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences). After blocking the nitrocellulose membranes in ODYSSEY block buffer for 1 h, membranes were incubated with primary Abs at 4°C overnight and then with IRDye 680/800–labeled IgG secondary Ab at room temperature for 1 h. Finally, fluorescence intensity was detected on the membranes with the LI-COR Infrared Imaging System and analyzed using ODYSSEY software.

**Statistical analysis**

Statistical analysis was performed using the two-tailed Student t test (unpaired) for determining differences between means in the chemotaxis, calcium flux, and tissue microarray assays. Data were expressed as mean ± SEM. The significant differences between groups are represented by *0.01 < p < 0.05, **0.001 < p < 0.01, and ***p < 0.001. For the chemotaxis assay, every chemoattractant protein was tested in triplicate wells, and the significant differences in chemoattractant activity between two proteins were evaluated in Prism 5.0 (GraphPad Software). In the calcium flux assay, several cells (n > 8) were randomly selected in each image, and the optical cell density in a series of images from 0 to 350 s was evaluated by the Leica System Analysis Software. The calcium-flux change curve generated from the cells in one image over time was analyzed in Prism 5.0 (GraphPad Software).

**Results**

**The expression, purification, and analysis of recombinant human PSMP**

The pcDB-PSMP plasmid was transiently transfected into the HEK293T cell line using the polyethyleneimine system. The mature secreted PSMP-myc-his recombinant protein was successfully secreted into the culture medium of PC3, DU145, MCF7, and MDA-MB-231 cell lines (ATCC) was evaluated by ELISA using anti-PSMP Abs. Rabbit polyclonal anti-PSMP was used as the capture Ab, and mouse monoclonal anti-PSMP, 3D5, was used as the detection Ab. The capture Ab was coated overnight in coating buffer. After blocking in 5% BSA for 30 min, cell supernatant was added and incubated with the capture Ab for 2 h. The supernatant was discarded, and samples in wells were washed five times with PBS containing 0.05% Tween 20 (PBST). The detection Ab was then added for 2 h. After another five washes with PBST, the HRP-conjugated rabbit anti-mouse IgG (H + L) was added as the secondary Ab for 30 min. After six washes with PBST, the samples were developed using the diaminobenzidine system.

**Western blot**

Cell lysates were extracted with RIPA lysis buffer and cleared by centrifugation at 16,000 × g for 15 min at 4°C. Total protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences). After blocking the nitrocellulose membranes in ODYSSEY block buffer for 1 h, membranes were incubated with primary Abs at 4°C overnight and then with IRDye 680/800–labeled IgG secondary Ab at room temperature for 1 h. Finally, fluorescence intensity was detected on the membranes with the LI-COR Infrared Imaging System and analyzed using ODYSSEY software.
expressed by HEK293T cells and could be detected in the supernatant (Fig. 1A). The molecular mass of the recombinant PSMP secreted into the supernatant was ~25 kDa by Western blot analysis, whereas the PSMP protein detected in the lysate was ~18 kDa (Fig. 1A). The purified PSMP protein exhibited a purity of ≥90%, as assessed by the OD assay (Fig. 1B). Furthermore, BFA treatment inhibited the ability of HEK293T to secrete PSMP (Fig. 1C), supporting the hypothesis that PSMP is secreted by the classical endoplasmic reticulum–Golgi pathway. We next analyzed the purified PSMP secreted protein for the location of the N-terminal signal peptide cleavage site. By N-terminal amino acid analysis, we found that the signal peptide is located at 0–36 residues in this secreted PSMP protein (Fig. 1D), which is consistent with the prediction made by SignalP-HMM software.

PSMP can chemoattract PBMs and PBLs

During the early screening process, PSMP-containing culture supernatant from HEK293T cells could induce PBMC migration. PSMP was determined to be the chemoattractant factor in supernatant, as purified recombinant PSMP protein induced PBMC migration. We further separated human peripheral blood into its PBM, PBL, and PMN components and found that both PBMs (Fig. 1E) and PBLs (Fig. 1F) were also chemoattracted by PSMP. However, PSMP was not able to induce PMN migration (Fig. 1G).

CCR2B is the chemotactic receptor for PSMP

To identify the functional receptor binding to PSMP, we transiently expressed the classical chemotactic receptors in HEK293 cells. We then evaluated the ability of PSMP to induce chemotaxis in the...
HEK293 cells expressing different receptors and found that PSMP was able to chemoattract CCR2B-expressing HEK293 cells (Fig. 2A). The known chemokines, CCL2 and CCL8, are also CCR2 ligands. HEK293 transiently expressing CCR5, another receptor for CCL8 and CCR1, one of whose ligand is CCL5, was not chemoattracted by PSMP (Fig. 2B, 2C), compared with CCL8 and CCL5. We further demonstrated that PSMP could desensitize the ability of CCL2 to chemoattract HEK293-CCR2B (Fig. 2D). Correspondingly, CCL8 and PSMP could cross-desensitize each other’s ability to chemoattract HEK293-CCR2B (Fig. 2E). To further evaluate the relationship between PSMP and CCR2B, we prepared mouse mAbs targeting PSMP and found that 3D5 and 4E7 could effectively neutralize PSMP chemotactic ability toward CCR2B-expressing HEK293 cells (Fig. 2F). Because PTX-sensitive G-proteins are known to play an essential role in chemotaxis induced by CCL2 (15, 16), we examined whether the ability of PSMP to chemoattract CCR2B also required such G-proteins. We therefore applied PTX, an inhibitor of Gi/o, to the PSMP-based chemotaxis assay. Fig. 2G showed that PTX abolished chemotaxis with similar efficacy between PSMP and CCL2, suggesting that chemotaxis induced by PSMP involves the Gi/o pathway. Furthermore, we tested the THP-1 cell line, which highly expresses endogenous CCR2B, in the PSMP-mediated chemotaxis assay. Indeed, PSMP could also chemoattract THP-1, similar to CCL2 and its ability to chemoattract THP-1, at all concentrations, which could be blocked by the pretreatment of 5 μM RS102895, a specific CCR2B inhibitor (Fig. 2H). PBMCs highly express a number of endogenous chemokine receptors, including CCR2B. RS102895 effectively blocked the chemotactic ability of PSMP to PBMCs (Fig. 2I), indicating that CCR2B was required for PSMP to chemoattract PBMCs.

**PSMP induces CCR2B internalization in CCR2B-expressing HEK293 cells**

G-protein-coupled receptors undergo agonist-dependent desensitization and internalization. To explore whether PSMP can induce CCR2B internalization, we used the recombinant 0.1 and 1 μM PSMP to stimulate, for 1 h, HEK293 cells transiently expressing CCR2B-EGFP. Both 0.1 and 1 μM PSMP stimulation could induce CCR2B-EGFP internalization, compared with equivalent doses of the BSA control, as assessed by confocal immunofluorescence; CCL2, used as a positive control, also showed a similar effect (Fig. 3A). In addition, compared with 0.1 μM of CCL2 or PSMP stimulation, 1 μM of CCL2 or PSMP stimulation induced a higher rate of CCR2B-EGFP internalization in a dose-dependent manner (Fig. 3A). Consistent with these results, a flow cytometric assay showed that CCR2B expression levels on the surface of HEK293 cells were reduced after 1 h of PSMP stimulation, similar to CCL2 (Fig. 3B). After four repeated experiments, the rate of internalization of CCR2B was calculated. We found that both 200 ng/ml CCL2 and 1000 ng/ml PSMP could obviously induce the internalization of CCR2B (Fig. 3C).

**PSMP induces CCR2B-mediated calcium flux in CCR2B-expressing HEK293 cells**

CCL2 stimulation has been reported to induce elevated intracellular calcium levels (17). To examine whether PSMP also affected in-
tracellular calcium levels, we carried out the calcium flux assay in CCR2B-expressing HEK293 cells and found that recombinant PSMP could induce calcium flux in CCR2B-expressing HEK293 cells in a dose-dependent manner (Fig. 4A, 4C, 4E), similar to CCL2 and CCL8. Furthermore, PSMP, CCL2, and CCL8 could cross-desensitize calcium flux induced by each other (Fig. 4A–D).

Radioactive binding assay further indicates that CCR2B is the receptor for PSMP

To perform radioactive binding assay analysis of PSMP and CCR2B binding, we established an HEK293 cell line stably expressing CCR2B-EGFP. The recombinant mature PSMP was labeled with $^{[125\text{I}]}$, and a PSMP-CCR2B binding curve (Fig. 5A) was obtained.

**FIGURE 4.** PSMP can induce calcium flux in HEK293 cells expressing CCR2B. (A–D) HEK293-CCR2B cells were loaded with 10 μM fluo-3 for 1 h. After cells were washed with PBS and images of the unstimulated state were obtained by confocal microscopy, cells were stimulated by CCL2 (100 nM), CCL8 (100 nM), or PSMP (200 nM) and constantly observed for 3 min. The second stimulation was then added, and the cells were constantly observed for another 3 min. The fluorescence intensity of the cells before and after the first and second stimulation was evaluated and analyzed by the Leica System Analysis Software. Scale bars, 200 μm. (E) After CCL2 (100 nM), CCL8 (100 nM), or PSMP (100, 200, or 500 nM) stimulation, the ratio of the maximum intensity and basal intensity of calcium flux was measured in the fluo-3–loaded HEK293-CCR2B cells.
by reacting $^{[125]}$I-PSMP with varying quantities of CCR2B-EGFP-HEK293 membrane extract. We found that $^{[125]}$I-PSMP binding could saturate CCR2B-EGFP-HEK293 cells. The dissociation constant $K_d$ was 1.4 nM, and $^{[125]}$I-PSMP binding could be competitively inhibited with increasing concentrations of unlabeled PSMP or CCL2 (Fig. 5B). The fMLF chemokine did not compete for $^{[125]}$I-PSMP binding to CCR2B (Fig. 5B). These results suggest that both PSMP and CCL2 can bind to CCR2B.

**Tissue and cell line distribution of PSMP expression**

On the basis of the DNA coding sequence of PSMP published on PubMed, we designed primers that can specifically identify the PSMP sequence, and PSMP mRNA levels were evaluated in tissues from the tissue library using real-time PCR. In general, PSMP was expressed at low levels in normal human tissue (Fig. 6A) and cell lines (Fig. 6B). To evaluate PSMP expression in cancerous or inflamed tissue by immunohistochemistry, we prepared rabbit anti-PSMP Abs by immunizing rabbits with prokaryotic PSMP protein. PSMP was more abundantly expressed in BPH than in prostate cancer (Fig. 6C, 6D), as assessed by an immunohistochemistry-based tissue microarray assay. The microarray (OD-CT-UrPrt03-001) was obtained from Shanghai Outdo Biotech (http://www.superchip.com.cn), and the PSMP expression was also detected in breast cancer tissue (Fig. 6C). In PC3 prostate cancer cell lines, PSMP also exhibited relatively high expression (Fig. 6B, 6E-G), which is consistent with a previous study (12). We also evaluated PSMP protein expression in the lysates from PC3, DU145, MCF7, and MDA-MB-231 cell lines by Western blot analysis and found that PSMP expression was detected in all four cell lines (Fig. 6E). Furthermore, the secreted PSMP form was found in PC3 cell supernatants by Western blot (Fig. 6F) and ELISA (Fig. 6G).

**PSMP upregulates phosphorylated ERK in the PC3 cell line**

Because we know that PSMP and CCR2 are both highly expressed in PC3 cells, we wanted to know whether PSMP had any influence on cancer cell proliferation or migration. p-ERK, an important molecule in cancer cell proliferation and migration, was detected in PSMP- or CCL2-stimulated PC3 cells, in which p-ERK was upregulated within 15 and 30 min after stimulation (Fig. 7A). Moreover, purified PSMP was pretreated with mouse IgG (as a negative control) or neutralizing anti-PSMP Abs (3DS or 4E7) for 30 min and used to stimulate PC3 cells for 15 min. Although control IgG-pretreated PSMP could still upregulate p-ERK, the neutralized PSMP could no longer upregulate p-ERK (Fig. 7B). Uregulated p-ERK was also prevented in PC3 cells pretreated for 30 min with the CCR2B inhibitor, RS102895, before PSMP stimulation (Fig. 7B). These results indicate that PSMP can upregulate p-ERK in PC3 cells by binding to CCR2B.

**Discussion**

Since the completion of the Human Genome Project, a large number of gene functions remain unknown. According to H-Invitational Database (H-InvDB) statistics, 35,631 genes are predicted to be protein-encoding genes, but the function for more than half of these potential protein-encoding genes has not yet been annotated (18). At present, the quest to identify all human proteins is proceeding worldwide, which will be important for finding novel candidate targets for medical care and drug development.

The chemokine superfamily was discovered during the past two decades. So far, 48 chemokines have been identified in humans, which bind to ~ 20 chemokine receptors (1). Moreover, a group of proteins with CLF has been found to signal through chemokine receptors (9, 11, 19). We adopted the PBMC chemoattractant platform to screen for unknown proteins with chemokine function, and found that PSMP—a protein with unknown function—exhibited chemotactic ability. A previous report indicated that PSMP was a protein secreted from the PC3 cell line (12). In the current study, we found that the purified recombinant PSMP protein exhibits chemotactic ability to induce migration in PBMs and PBLs, but not PMNs, indicating that PSMP is a chemoattractant protein and may play some role in inflammation. CCR2B was found to be the receptor mediating PSMP chemotaxis by screening HEK293 cells expressing the classical chemokine receptors in a Boyden chamber assay. This finding was confirmed by in vitro chemotaxis, receptor internalization, calcium flux, and RBA assays. The $K_d$ of $^{[125]}$I-PSMP and CCR2B binding interaction was 1.4 nM, which is similar to the $K_d$ exhibited by CCL2 binding to CCR2B (20). Taken together, these results demonstrated that the functional effects displayed by PSMP are similar to those of CCL2, a known CCR2B agonist, indicating that PSMP functions as another potent CCR2B agonist.

The classical chemokine superfamily members have been characterized by their CC, CXC, CX3C, or XC structures, which are defined as an immunocyte-attracting motif (2). Although their sequence homology is highly variable, ranging from ∼20% to > 90%, they all share very similar tertiary structures (21). The mature PSMP protein contains CXC and CC motifs in 10 cysteines, which is different with the characteristic sequences contained in classical chemokine structures. Sequence homology analysis of PSMP shows that PSMP is homological to β-microseminoprotein, for which MSMP was named. PSMP shows no homology to any known chemokines, including CCL2 and CCL8. Recently, several proteins were found to exhibit chemotactic ability without containing the classical CC, CXC, CX3C, or XC characteristic structures, but were similar to classical chemokines in their three-dimensional spatial structures and could bind to chemokine receptors; these proteins included MIF (22), β-defensins (10), and a tyrosyl-tRNA-synthetase fragment (11). In one recent study, the
threading technique was applied to predict the structural characteristics and function of an unknown protein. The putative new chemokine from this study, DMC, was found by chemokine-related functional experiments. On the basis of these studies, DMC was predicted to contain an IL-8–like chemokine fold and to be structurally and functionally related to CXCL8 and CXCL14 (23). Some CLF polypeptides have been postulated to share tertiary structural features with corresponding canonical chemokine ligands, enabling them to use chemokine receptors; for example, MIF shares similar tertiary structural features with CXCL8 (9). However, the structural features of PSMP binding to the CCR2B receptor still need further research.

Recently, a study found that CCL2 could recruit inflammatory monocytes to facilitate prostate and breast tumor growth; CCL2 likely exerts its protumorigenic effects through recruiting tumor-associated macrophages, as these macrophages promote a tumorigenic microenvironment through inducing production of growth enhancers, angiogenic factors, and inflammatory mediators (24–26). In other recent studies, CCR2-related signaling pathways, including Ras/Raf/MEK/ERK (27) and PI3K/AKT (28), partici-

FIGURE 6. PSMP is highly expressed in BPH and can be detected in prostate cancer and breast cancer. (A) PSMP mRNA expression levels in normal tissues from the tissue library were measured by real-time PCR using specific primers. (B) The transcriptional level of PSMP in multiple cancer cell lines was evaluated by RT-PCR using specific primers. (C) PSMP protein expression levels were detected in BPH, prostate cancer, and breast cancer by immunohistochemistry using rabbit anti-PSMP polyclonal Ab. Scale bars, 500 μm (upper row) and 100 μm (lower row). (D) PSMP expression in prostate cancer and BPH was measured and counted (***p = 0.0009) in the tissue microarray by immunohistochemical staining using rabbit anti-PSMP polyclonal Ab. (E) PSMP protein expression was evaluated by Western blot using rabbit anti-PSMP polyclonal Ab in PC3, DU145, MCF7, and MDA-MB-231 cell lysates. (F) The secreted PSMP protein was detected in PC3 supernatant by Western blot using rabbit anti-PSMP polyclonal Ab. (G) After culturing in Hektor G media for 3 d, the secreted PSMP protein levels in the supernatant from PC3, DU145, MCF7, and MDA-MB-231 cells were evaluated by ELISA.
The authors have no financial conflicts of interest.

Acknowledgments

We thank Dr. Philip M. Murphy (Laboratory of Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health) for kindly providing CCR1, CCR3, and CCR8 expression plasmids and Dr. Lan Duan (Beijing Red Cross Blood Center) for help.

Disclosures

FIGURE 7. PSMP upregulates p-ERK in the PC3 cell line. (A) After starving PC3 cells in RPMI 1640 for 12 h, PC3 cells were stimulated by PSMP (200 ng/ml) or CCL2 (100 ng/ml) for 0, 15, 30, or 60 min. The p-ERK and ERK1/2 levels in the PC3 cell lysate were detected by Western blot. (B) Before stimulating PC3 cells with PSMP, the PSMP protein was treated with mouse mAbs against PSMP (3D5 or 4E7), or PC3 cells were treated with the CCR2B inhibitor RS102895. The p-ERK and ERK1/2 levels were detected by Western blot after stimulation.

References