A Pectic Polysaccharide Isolated from the Roots of Bupleurum falcatum L. Stimulates the Tyrosine Phosphorylation of Lipid Rafts of Murine B Cells

Tsukasa MATSUMOTO,a,b,# Kanako HOSONO-NISHIYAMA,a,# and Haruki YAMADA*,a,b

a The Kitasato Institute for Life Sciences and Graduate School of Infection Control Sciences, Kitasato University; Minato-ku, Tokyo 108–8641, Japan; and b Oriental Medicine Research Center, The Kitasato Institute; Minato-ku, Tokyo 108–8642, Japan. Received January 8, 2008; accepted January 24, 2008.

Bupleuran 2IIc, a pectic polysaccharide isolated from the roots of Bupleurum falcatum L., was previously characterized as a T cell-independent B cell mitogen. The endo-(1→4)-α-d-polygalacturonase-resistant moiety of bupleuran 2IIc (bupleuran 2IIc/PG-1) was the active site for expression of the activity, and expression of the cyclin D2 gene by bupleuran 2IIc/PG-1 may be mediated via activation of Src family tyrosine kinase, phosphatidylinositol 3-kinase (PI 3-K) and phospholipase C (PLC)-γ followed by activation of protein kinase C (PKC) and calcium mobilization (Matsumoto et al., Int. Immunopharmacol., 5, 1373–1386 (2005)). Plasma membrane microdomains (lipid rafts) are enriched in signaling molecules and suggested to be involved in numerous cell functions, including membrane traffic and signaling. When B cells were stimulated with bupleuran 2IIc/PG-1, clustering of membrane lipid rafts was observed. To consider whether lipid rafts are implicated in bupleuran 2IIc/PG-1-mediated B cell proliferation, we analyzed the phosphorylation of tyrosine residues of proteins in lipid rafts. When murine B cells were stimulated with bupleuran 2IIc/PG-1, tyrosine phosphorylation of proteins in lipid rafts fraction was observed within 5 min. Tyrosine phosphorylation in lipid rafts fraction by bupleuran 2IIc/PG-1 was inhibited by the Src-family tyrosine kinase inhibitor, PP2. Together with previously published data, the results presented in this study suggest that activation of signaling molecules in lipid rafts by stimulation of bupleuran 2IIc/PG-1 contributes to B cell proliferation as the membrane-proximal signaling event.

Key words pectic polysaccharide; Src family tyrosine kinase; B cell; lipid raft

The roots of Bupleurum falcatum L. have been used extensively as Chinese and Japanese herbal medicines. The pharmacologically active polysaccharides, bupleuran 2IIb and 2IIc, have been isolated from hot water extract of the roots of B. falcatum. Bupleuran 2IIb has been characterized as a pectin-like polysaccharide consisting of a galacturonan region consisting of a rhamnogalacturonan core with neutral sugar side chains and a rhamnogalacturonan II-like region. Bupleuran 2IIc has been characterized as a T cell-independent B cell mitogen that induces proliferation and maturation of murine B cells, which differentiate into immunoglobulin (Ig)-secreting plasma cells by a T cell-independent mechanism. Bupleuran 2IIc has been shown to stimulate secretion of interleukin (IL)–6 in murine B cells, which differentiate into immunoglobulin (Ig)-secreting plasma cells by a T cell-independent mechanism.

In mammalian cells, the D-type cyclins are considered the endpoint targets of mitogenic signaling pathways and function as growth factor sensors. Previous studies have shown that the endo-(1→4)-α-d-polygalacturonase-resistant moiety of bupleuran 2IIc (bupleuran 2IIc/PG-1) is the active site for B cell activation, and expression of the cyclin D2 gene by bupleuran 2IIc/PG-1 may be mediated via activation of Src family tyrosine kinase, phosphatidylinositol 3-kinase (PI 3-K) and phospholipase C (PLC)-γ followed by activation of protein kinase C (PKC) and calcium mobilization. However, the molecular basis of the membrane-proximal signaling event for the proliferation response of B cells to stimulation by bupleuran 2IIc remains unknown.

The plasma membrane provides both a barrier and an interface to the extracellular microenvironment and supports many cell surface proteins responsible for transmitting extracellular signals to the cell interior. Recent biochemical, pharmacological, and imaging data indicate that cell plasma membranes contain microdomains, distinct from the bulk plasma membrane and caveolae, which are frequently referred to as lipid rafts. Studies of lipid rafts isolated from various cells have suggested that they are enriched for certain protein species, including glycosylphosphatidylinositol (GPI)-linked proteins, fatty-acylated proteins, receptors, and cytoskeletons, as well as many proteins known to be important for signal transduction such as Src-family tyrosine kinases.

In an attempt to elucidate the mode of action of bupleuran 2IIc/PG-1 on the membrane-proximal signaling linked to B cell activation, we analyzed tyrosine phosphorylation in lipid rafts fraction. To our knowledge, this is the first study of lipid rafts linked to B cell activation by stimulation of mitogenic pectic polysaccharide.

MATERIALS AND METHODS

Animals Specific pathogen-free C3H/HeJ (H-2k) female mice (6–8 weeks old) were obtained from SLC (Shizuoka, Japan). The mice were maintained in a 24-h light and dark cycle (12 h of light, 12 h of darkness) and controlled temperature (23 ± 1 °C) and had free access to standard laboratory chow (CE-2, CLEA Japan Inc., Tokyo, Japan) and water. The procedure from the Prime Minister’s Office of Japan (No. 6 of March 27, 1980) for the care and use of laboratory animals was followed. The experiments were conducted in accordance with the Guidelines for Animal Use and Experimentation of the Kitasato Institute (Tokyo, Japan), and the approval number of the animal experimentation was 2007-1-35-1 (Kitasato Institute).

Reagents Phosphate-buffered saline (PBS), RPMI 1640 medium, horseradish peroxidase (EC 1.11.1.7, HRP)-conju-
gated choleratoxin B subunit (CTB), FITC-conjugated CTB, protease inhibitor cocktail, and phosphatase inhibitor cocktail 2 were purchased from Sigma (St. Louis, MO, U.S.A.). PP2 and PP3 were obtained from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). Mouse anti-phosphotyrosine mAb (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). Rabbit polyclonal anti-PLCγ2 IgG (Q-20) and mouse anti-Lyn mAb (H-6) were purchased from Santa Cruz Biotechnology (Delaware, CA, U.S.A.). Cell-surface labeling was performed with sulfo-NHS-LC-biotin (Pierce, Rockford, IL, U.S.A.).

**Preparation of Bupleuran 2IIc/PG-1** Bupleuran 2IIc was purified as described previously.1,13 Bupleuran 2IIc/PG-1 (rhamnogalacturonan core possessing side chains rich in neutral sugars) was prepared from bupleuran 2IIc by endo-(1→4)-α-L-galacturonase (EC 3.2.1.15) digestion followed by gel filtration as reported previously.2,3 The proposed structure of bupleuran 2IIc/PG-1, which was previously reported by our group,2—4 is shown in Fig. 1.

**Preparation of B Cells from Spleen and Determination of Cell Viability** Preparation of B cells from spleen was performed using anti-mouse CD43 (Ly-48) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously.14 The cell fraction was shown to contain >95% CD45R/B220 and CD19 double-positive B cells (data not shown). Cell viability was determined by trypan blue exclusion, and that just after the preparation was uniformly >98%.

**Preparation of Lipid Rafts Fraction** B cells (1×10^8 cells) were lysed in TNE buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA) with 1% Triton X-100, protease inhibitor cocktail, and phosphatase inhibitor cocktail 2 on ice. The lysate including insoluble materials was further homogenized with 10 strokes in a Teflon glass homogenizer and centrifuged at 900 g at 4°C for 10 min. The cell extracts were mixed with an equal volume of 85% sucrose in TNE buffer and layered at the bottom of a Beckman centrifuge tube (14×89 mm; Beckman instruments, Inc., Spino Divsion, CA, U.S.A.). The lysates were overlayered with a discontinuous sucrose density gradient (6 ml 35% sucrose, then 3.5 ml 5% sucrose in TNE buffer). After centrifugation at 200000 g at 4°C for 20 h, 1.0-ml fractions were collected from the top to the bottom of gradients. Finally, fraction 4 containing the lipid rafts was diluted with TNE buffer, and centrifuged at 200000 g at 4°C for 1 h to pellet and concentrate the rafts.

**Immunoblotting** Samples were electrophoresed through 10% or 12.5% SDS-PAGE gel, and the proteins were blotted onto nitrocellulose membranes (Hyper film™, Amersham Pharmacia Biotech, Buckinghamshire, U.K.), probed with different antibodies, followed by enhanced chemiluminescence detection using ECL Reagent (Western Lightning™, PerkinElmer Life Sciences, Boston, MA, U.S.A.). Figures of autoradiographs were constructed using CanoScan 8400FV (Canon, Tokyo, Japan) and Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA, U.S.A.).

**Immunoprecipitation** Lipid rafts were precleared with Protein G Sepharose® 4 Fast Flow (Amersham Pharmacia Biotech) and the resulting samples were incubated at 4°C for 60 min with 5 μg of the antibodies to be tested. Immune complexes were precipitated with Protein G Sepharose® 4 Fast Flow during a 20-h incubation at 4°C and were washed extensively with lysis buffer before solubilization by heating in reducing Laemmli buffer.

**Fluorescence Microscopy** B cells were stimulated with bupleuran 2IIc/PG-1 (100 μg/ml) at 37°C for indicated time, then the cells were fixed by 3% formalin in PBS at room temperature for 30 min. The cells were washed with PBS and stained with FITC-conjugated CTB (10 μg/ml) at 37°C for 10 min, and fluorescent images were digitally recorded.

**RESULTS AND DISCUSSION**

Plasma membrane rafts act as platforms on the cell surface integrating signaling pathways.15 Because clustering (capping) of the lipid rafts has been reported to be important for cell surface receptor-mediated signaling, we examined whether clustering of lipid rafts on B cells was induced by stimulation of bupleuran 2IIc/PG-1. To detect the membrane lipid rafts microdomains, B cells were stained with CTB, which binds to the ganglioside GM1, a component of lipid raft in the outer leaflet of the plasma membrane.9,10 The cells were analyzed by fluorescence microscopy to determine the localization of GM1 in the cell membrane. When B cells were stimulated with bupleuran 2IIc/PG-1, clustering of lipid rafts was observed (Fig. 2), suggesting that bupleuran 2IIc/PG-1 activates B cells via lipid rafts-mediated signaling system.

Phosphorylation of tyrosine residues of proteins by specific tyrosine kinases is the major process of signaling pathways. It is of interest to determine whether stimulation of B cells with bupleuran 2IIc/PG-1 could induce protein phos-
phorylation in lipid rafts. To investigate tyrosine phosphorylation of proteins in lipid rafts in response to bupleuran 2IIc/PG-1, B cells were fractionated and lipid rafts fraction was obtained by flotation through discontinuous sucrose gradients using detergent-based (Triton X-100) methods. To confirm the location of lipid rafts on gradients, 10 μl of each fraction was blotted onto nitrocellulose membranes and probed by blotting for the presence of ganglioside GM1 (GM1) using HRP-conjugated CTB. GM1 was exclusively detected in fraction 4 (Fig. 3B), which included the 5%/35% sucrose interface where flocculent material was visible (Fig. 3A), indicating that fraction 4 contained lipid rafts. When gangliosides were extracted from lipid rafts and analyzed by high-performance TLC, the presence of GD1a and GD1b in addition to GM1 was noted (data not shown). When cell lysates prepared from biotinylated B cells were fractionated, most of the biotinylated proteins were liberated with 1% Triton X-100 from the plasma membrane and recovered mainly in heavy-density soluble fractions (Fig. 3C). A small amount of proteins was recovered in fraction 4 (Fig. 3C), which included the lipid rafts, suggesting that lipid rafts contain cell surface proteins.

Tyrosine phosphorylation of proteins in the lipid rafts fraction was investigated by Western blotting using an anti-phosphotyrosine-specific antibody (Fig. 4A). Two heavily phosphorylated protein bands at 50—60 kDa and 70—80 kDa were observed in lipid rafts from unstimulated B cells (Fig. 4A). When B cells were stimulated with bupleuran 2IIc/PG-1, increased tyrosine phosphorylation was observed in lipid rafts fraction after 5-min stimulation on several substrates (Fig. 4A, arrowheads), indicating the movement into rafts of phosphorylated proteins and/or the phosphorylation of resident proteins within lipid rafts by activated kinases. Whereas, no measurable tyrosine phosphorylation was observed in heavy-density soluble fraction (Fig. 4B). From these results, it was assumed that early signaling events were targeted to lipid rafts. In deed, recruitment of PLCγ2 into lipid rafts fraction after stimulation of B cells with bupleuran 2IIc/PG-1 was observed in a preliminary experiment (Fig. 4C).

The phosphorylated proteins of 50- to 60-kDa in lipid rafts are likely to include Src-family protein kinases, because it has been reported that Src-family tyrosine kinases such as Lyn are enriched in lipid rafts of B cells. Next, we exam-
ined whether Lyn, a 53/56-kDa signaling regulator protein, in lipid rafts was phosphorylated by stimulation of bupleuran 2IIc/PG-1. After stimulation of B cells with or without bupleuran 2IIc/PG-1, Lyn was immunoprecipitated by the use of anti-Lyn antibody from the lipid rafts, and was analyzed by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-phosphotyrosine or anti-Lyn antibody. The amount of precipitated Lyn from lipid rafts fraction was not changed by the stimulation of B cells with bupleuran 2IIc/PG-1 (Fig. 5A lower panel). However, when B cells were stimulated with bupleuran 2IIc/PG-1, markedly enhanced phosphorylation of Lyn in lipid rafts was observed (Fig. 5A upper panel). The stimulation with bupleuran 2IIc/PG-1 induced clustering of lipid rafts (Fig. 2). It has been reported that Src-family tyrosine kinases are activated via lipid rafts clustering-mediated autophosphorylation followed by phosphorylation of their substrates and thus triggering of signaling cascades. To determine whether Src-family tyrosine kinases contributed to the tyrosine phosphorylation of lipid rafts observed following bupleuran 2IIc/PG-1 stimulation, the effect of PP2, a specific inhibitor of the Src family of tyrosine kinases, was examined. Bupleuran 2IIc/PG-1-induced tyrosine phosphorylation in lipid rafts fraction was markedly decreased by PP2 and not by PP3, an inactive analogue of PP2, suggesting that Src-family of tyrosine kinase activity contributes to tyrosine phosphorylation of lipid rafts (Fig. 5B).

Together with previously published data suggesting that the cyclin D2 expression of B cells by bupleuran 2IIc/PG-1 is mediated via the activation of Src-family tyrosine kinases, the results presented in this study suggest that activation of Src-family tyrosine kinases of lipid rafts contributes to B cell proliferation by stimulation of bupleuran 2IIc/PG-1 as the membrane-proximal signaling event.

Acknowledgments We are grateful to Dr. Yuji Kikuchi for helpful discussion, and to Ms. Michiko Sekiya for her technical assistance. A part of this work was supported by a grant of the 21st Century COE Program from the Ministry of Education, Culture, Sports, Sciences, and Technology of Japan.

REFERENCES


Fig. 5. Src Family Tyrosine Kinase-Mediated Protein Tyrosine Phosphorylation

(A) Phosphorylation of Lyn after stimulation with bupleuran 2IIc/PG-1. B cells (1×10^6 cells) were harvested 5 min after stimulation with or without bupleuran 2IIc/PG-1 (100 μg/ml), and Lyn was immunoprecipitated (IP) by the use of anti-Lyn antibody from the lipid rafts, as described in Materials and Methods. The bound components were eluted by heating in Laemmli buffer, analyzed by 10% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-phosphotyrosine (anti-pTyr) (upper panel) or anti-Lyn antibody (lower panel) (B). (B) Effect of Src family tyrosine kinase inhibitor on bupleuran 2IIc/PG-1-induced protein tyrosine phosphorylation in lipid rafts. B cells (1×10^6 cells) were stimulated with bupleuran 2IIc/PG-1 (100 μg/ml) for 10 min in the presence or absence of a 30-min pretreatment with PP2 or PP3 (10 μM). Lysates were subjected to sucrose density gradients, as described in Materials and Methods. Phosphorylation of tyrosine of lipid rafts was monitored with anti-phosphotyrosine. Arrowheads indicate stimulation-induced tyrosine-phosphorylated proteins.