Trp-Lys-Tyr-Met-Val-Met stimulates phagocytosis *via* phospholipase D-dependent signaling in mouse dendritic cells

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Abstract

Dendritic cells (DCs) play a key role in activating the immune response against invading pathogens as well as dying cells or tumors. Although the immune response can be initiated by the phagocytic activity by DCs, the molecular mechanism involved in this process has not been fully investigated. Trp-Lys-Tyr-Met-Val-Met-NH$_2$ (WKYMVM) stimulates the activation of phospholipase D (PLD) *via* Ca^{2+} increase and protein kinase C activation in mouse DC cell line, DC2.4. WKYMVM stimulates the phagocytic activity, which is inhibited in the presence of N-butanol but not *t*-butanol in DC2.4 cells. Furthermore, the addition of phosphatidic acid, an enzymatic product of PLD activity, enhanced the phagocytic activity in DC2.4 cells. Since at least two of formyl peptide receptor (FPR) family (FPR1 and FPR2) are expressed in DC2.4 as well as in mouse bone marrow-derived dendritic cells, this study suggests that the activation of FPR family by WKYMVM stimulates the PLD activity resulting in phagocytic activity in DC2.4 cells.

Introduction

Dendritic cells (DCs) are well-known dedicated antigen presenting cells, and perform important roles in the regulation of the immune response (Guermonprez *et al.*, 2002; Granucci *et al.*, 2003). During the initiation of the immune response, DCs capture the invading pathogens, dying cells or tumor antigens. After antigen uptake, the DCs process and present the processed peptides on the surface of the DCs inducing the stimulation of naive T cells into antigen-specific immune responses (Guermonprez *et al.*, 2002; Granucci *et al.*, 2003). Since phagocytosis of the antigen by DCs is an initial step for the proper immune responses, it is important to reveal the regulation mechanism of this event. However, the regulation of phagocytic activity as well as the signaling pathways of the event in DCs has not been reported.

Phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA), has been reported to play an important role in the regulation of the immune response by phagocytic cells (Lennartz, 1999; Cockraft, 2001). The potential role(s) for the PLD-generated PA in biological functions of the phagocytic cells is suggested by virtue of the unique enzymatic characteristics of PLD *i.e.* the transphosphatidylation reaction by which the phosphatidyl moiety of the phospholipid is transferred to a primary alcohol to produce the phosphatidylalcohol at the expense of PA. The presence of the primary alcohol has been reported to interfere with the activation of NADPH oxidase and degranulation (Bae *et al.*, 1999; Giron-Calle and Forman, 2000; Kaldi *et al.*, 2002). Although two isoforms of PLD, PLD1 and PLD2, have been cloned in mammalian cells (Hammond *et al.*, 1995; Colley *et al.*, 1997), little is known regarding the molecular identity of the PLD isoforms expressed in DCs and their roles in activationg the DCs.

The formyl peptide receptor (FPR) family is one of the classical chemoattractant receptors, which are G protein-coupled, and seven trans-membrane receptors. The FPR family receptors have been reported to play an important role in modulating innate immune

responses in mice (Gao *et al.*, 1999; Hartt *et al.*, 2000; Liang *et al.*, 2000; Tiffany *et al.*, 2001). Activating FPR1 *via* its specific agonist, *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) induced the chemotactic migration of phagocytic cells, such as monocytes and neutrophils. The fMLF also has been reported to elicit superoxide generation in the mouse neutrophils *via* FPR1 activation (Fillion *et al.*, 2001; Li *et al.*, 2002). Regarding the functional roles of FPR2, the amyloid beta peptide has been reported to induce phagocyte chemotaxis and oxidant stress in phagocytes (Tiffany *et al.*, 2001). Although the functional roles of the activation of the FPR family receptors in the neutrophils were extensively studied, their roles in the DCs have not been fully investigated. Recently Yang *et al.* demonstrated that FPR and FPRL2 are expressed in human immature DCs or mature DCs (Yang *et al.*, 2002). FPRL1 was reportedly down-regulated in human immature DCs and mature DCs (Yang *et al.*, 2001; Yang *et al.*, 2002). On the functional role of FPR and FPRL2 in human DCs, the two receptors are involved in the chemotactic migration of DCs (Yang *et al.*, 2002). However, the expression and function of FPR family in mouse DCs have not been fully investigated until now.

Previously we reported on the synthetic peptide, Trp-Lys-Tyr-Met-Val-Met-CONH$_2$ (WKYMVM), which can stimulate leukocyte activity (Baek *et al.*, 1996). WKYMVM was found to stimulate phosphoinositide (PI) hydrolysis and extracellular signal-regulated protein kinase (ERK) activation in U937 cells, and these events proved to be PTX-sensitive, suggesting the involvement of PTX-sensitive G-protein (Baek *et al.*, 1999). Recently it was demonstrated that WKYMVM is a ligand for FPR family (FPRL1 and FPRL2 but not FPR) (Christophe *et al.*, 2001). In this study, we investigated the functional role of FPR family using a specific agonist, WKYMVM in the immature DCs, the DC2.4 cells. The major function of the immature DCs is the phagocytosis of pathogens, apoptotic cells, or tumor cells, and we focused on the modulation of phagocytic activity by WKYMVM.

Materials and Methods

Materials

WKYMVM was synthesized, purified, and prepared in the Peptide Library Support Facility at Pohang University of Science and Technology, as described previously (Baek *et al.*, 1996). Radioiodinated Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm) (Seo *et al.*, 1997) ([^{125}I]-labeled) and [9, 10 (n)-^{3}H] myristic acid (53 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). fMLF and fluores-

cein-conjugated dextran were purchased from Sigma (St. Louis, MO). The precoated silica gel TLC plates (F-254) were obtained from Merck (Darmstadt, FRG). The RPMI 1640 was acquired from Invitrogen Corp. (Carlsbad, CA). Dialyzed FBS and supplemented bovine calf serum were purchased from Hyclone Lab. Inc. (Logan, UT). Fura-2 pentaacetoxymethyl ester (fura-2/AM) and 1, 2-bis (2-aminophenoxy) ethane-*N, N, N', N'*-tetraacetoxymethylester (BAPTA/AM) were purchased from Molecular Probes (Eugene, OR). 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF109203X), {3-[1-[3-(amidinothio) propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimi de methane sulfonate (Ro-31-8220), and 2'-amino-3'-methoxyflavone (PD98059) were obtained from Calbiochem (San Diego, CA). The antibodies specific to PLD, C-terminal-specific antibodies, were prepared as described previously (Lee *et al.*, 1997).

Cell culture

The cell line DC2.4, derived from C57Bl/6 mice, was originally provided by Dr. Kenneth L. Rock (Division of Lymphocyte Biology, Dana Farber Cancer Institute, Boston, MA) and maintained as previously reported (Shen *et al.*, 1997). The DC2.4 cells have a dendritic morphology, express the dendritic cell-specific markers, the MHC molecules, the co-stimulatory molecules, and have phagocytic activity as well as antigen-presenting capacity (Shen *et al.*, 1997). The cells were maintained at approximately 1×10^6 cells/ml under the standard incubator conditions (humidified atmosphere, 95% air, 5% CO$_2$, 37°C). Primary mouse DCs were prepared as described previously (Shin *et al.*, 2003). Briefly, bone marrow cells were isolated form the femur and tibia of C57BL/6 mice and then, the bone marrow cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 50 μM 2-mercaptoethanol, 20 ng/ml murine GM-CSF and 10 ng/ml murine rIL-4 at 37°C in a humidified 5% CO$_2$ incubator. On day 3, non-adherent granulocytes and B and T lymphocytes were gently removed, and fresh media were added. On day 6 of culture, released, immature, non-adherent cells with the typical morphological features of DCs were harvested (Shin *et al.*, 2003).

Ligand binding assay

The ligand binding analysis was performed as described previously (Hu *et al.*, 2001). Briefly, the DC2.4 cells were seeded at 1×10^5 cells per well into a 24 well plate and cultured overnight. After blocking the cells with a blocking buffer (33 mM HEPES, pH 7.5, 0.1% BSA in RPMI 1640) for 2 h, several concen-

trations of the labeled WKYMVm was added to the cells in a binding buffer (PBS containing 0.1% BSA), in the presence or absence of the unlabelled peptides, and incubated for 3 h at 4°C with continuous shaking. The samples were then washed 5 times with ice-cold binding buffer, and 200 μl of a lysis buffer (20 mM Tris, pH 7.5, 1% Triton X-100) was added to each well. After 20 min at room temperature, the lysates were collected and counted using a γ-ray counter (Hu *et al.*, 2001).

RT-PCR analysis

The mRNA was isolated by using a QIAshredder and an RNeasy kit (Qiagen, Hilden, Germany). mRNA, M-MLV reverse transcriptase, and pd(N)^6 primers (Invitrogen, Carlsbad, CA) were used to obtain the cDNA. The primers used for the RT-PCR analysis have been reported previously (Wang and Ye, 2002). The sequences of the primer used are as follows; FPR1: sense, 5'-ACAGCCTGTACTTTCGAC-3'; anti-sense, 5'-CTGGAAGTTAGAGCCCGTTC-3'. FPR2: sense, 5'-GTCAAGATCAACAGAAGAAACC-3'; anti-sense, 5'-GGGCTCTCTCAAGACTATAAGG-3'. FPR-rs1: sense, 5'-GGCAACTCTGTTGAGGAAAG-3'; anti-sense, 5'-G-GCTCTCGGTAGACGAGA-3'. FPR-rs3: sense, 5'-GG-TGTCAATTACCGTGAGC-3'; anti-sense, 5'-CTCTCT-AAATTCTTGACCAAGAAAG-3'. FPR-rs4: sense, 5'-A-GATGTCTGTTATTGCTGCC-3'; anti-sense, 5'-CAGT-GTCTCATTGTTAAAGATGTTG -3'. FPR-rs5: sense, 5'-ACAATTAAAGGTATTCTTTACTGTGAAC-3'; anti-sense, 5'-GCGAATTCTCCTCAGGGCCTACTCTT-C-3'. FPR-rs6: sense, 5'-CCCCTGAGGAGCAAGT-AAAAGTAT-3'; anti-sense, 5'-CAGGGCTGAGTCCTC-CCTTA-3'. FPR-rs7: sense, 5'-CCTGAGGAGCAGGT-AAACATGT-3'; anti-sense, 5'-GGGCTGAATCCTCCC-TCA-3'. Actin: sense, 5'-ATGGATGATGATATCGCC-GCG-3', anti-sense, 5'-TCTCCATGTCGTCCCAGTTG-3'. Forty PCR cycles were run at 94°C (denaturation, 30 s), 61°C (annealing, 30 s), and 72°C (extension, 30 s). The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Measurement of intracellular calcium concentration

The intracellular calcium concentration ($[Ca^{2+}]_i$) was determined using fura-2/AM as described before (Bae *et al.*, 2001a). Briefly, the prepared cells were incubated with 3 μM fura-2/AM at 37°C for 50 min in fresh serum free RPMI 1640 with continuous stirring. 2×10^6 cells were aliquoted for each assay in Ca^{2+} free Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, and 0.2 mM EGTA). The fluorescence changes at the dual excitation wavelengths of 340 nm and 380 nm as well as the emission wavelength of 500 nm were measured. The calibrated fluorescence ratio was translated into $[Ca^{2+}]_i$.

Measurement of phosphatidylbutanol formation

The production of phosphatidylbutanol (PBt) was determined using a slight modification of the method previously reported (Bae *et al.*, 2001b; Kim *et al.*, 2003). The DC2.4 cells were seeded at 1×10^6 cells/ ml in RPMI 1640 containing 10% FBS, and the cells were cultured for 20 h with serum free RPMI1640. The cells were then loaded with [^{3}H]-myristic acid (5 μCi/ml) for 4 h at 37°C. The loaded DC2.4 cells were then washed twice with serum-free RPMI 1640 and stimulated with the peptide in the presence of 0.5% n-butanol. After 30 min, the reactions were quenched by adding 0.5 ml ice-cold methanol, which was followed by aspirating the medium. After adding 0.5 ml of chloroform and 0.5 ml of 1 M NaCl, the total lipids were extracted by vigorous vortexing. The lower phase obtained after centrifugation at 550 *g* for 10 min was dried under nitrogen gas. The lipids were then dissolved in chloroform: methanol (95:5), spotted onto silica gel 60 TLC plates, and separated using a solvent containing chloroform: methanol: acetic acid (90: 10: 10) as described previously (Bae *et al.*, 2001b). In order to determine the quantity of PBt and the total lipids, a Fuji BAS-2000 image analyzer (Fuji Film Co., LTD) was used.

Immunoprecipitation

The cultured DC 2.4 cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.2, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1% cholic acid, 1 mM Na_3VO_4, 50 mM NaF, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonylfluoride), and proteins were harvested by centrifugation at 4°C for 5 min at 20,000 *g*. For immunoprecipitation, protein A-Sepharose coupled to anti-PLD antibody (5 μg), which is specific to C-terminal of PLD and recognizes both PLD1 and PLD2 (Lee *et al.*, 1997), was incubated with the cell lysate (2 mg) for 4 h at 4°C. The immune complexes were then washed five times with lysis buffer, and the immunoprecipitated proteins were released by heating at 95°C for 5 min in Laemmli sample buffer before electrophoresis and immunoblot analysis with anti-PLD antibodies: C-terminal-specific antibodies.

Electrophoresis and immunoblot analysis

The protein samples were prepared for electrophoresis. The proteins in the samples were then separated in an 8% SDS-polyacrylamide gel using the buffer system described by Laemmli (King and Laemmli, 1971; Kim *et al.*, 2003). Following electro-

phoresis, the proteins were blotted onto a nitrocellulose membrane, which were then blocked by incubating them with TBST (Tris-buffered saline, 0.05% Tween-20) containing 5% non-fat dry milk. Subsequently, the membranes were incubated with the specific antibodies indicated and then washed with TBST. The antigen-antibody complexes were visualized after incubating the membrane with 1:5,000 diluted goat anti-rabbit IgG or goat anti-mouse IgG antibodies coupled with horseradish peroxidase. The complexes were then detected by enhanced chemiluminescence.

Measurement of phagocytosis of FITC-dextran by DCs

The phagocytic activity of the DCs was measured as described previously (Xia and Kao, 2002). DC2.4 cells (1×10^5) were incubated with 1 mg/ml of FITC-dextran in 500 µl of PBS containing 1% human serum at $37^{o}C$ for various lengths of time periods. The reaction was quenched by adding 2 ml of ice-cold PBS containing 1% human serum and 0.02% sodium azide. The samples were washed three times with ice-cold PBS and analyzed by using a FACScan flow cytometer.

Results

DC2.4 cells express cell surface receptor(s) for WKYMVm

Initially, the expression of FPR family proteins in the DC2.4 cells was investigated by using [125I]-labeled WKYMVm, a specific ligand for FPR family (FPR, FPRL1, and FPRL2) (Le *et al.*, 1999; He *et al.*, 2000; Christophe *et al.*, 2001). The DC2.4 cells were incubated with various concentrations of [125I]-labeled WKYMVm in the presence or absence of excess of the unlabeled WKYMVm, WKYMVM, or fMLF. As shown in Figure 1A, the specific binding of [125I]-labeled WKYMVm was observed showing the saturation mode. When the ligand binding assays were performed in the presence of several concentrations of the unlabeled WKYMVM, or fMLF, specific binding of the [125I]-labeled WKYMVm was partially inhibited concentration-dependently (Figure 1B). However, the addition of a combination of unlabeled WKYMVM and fMLF caused an inhibition of [125I]-labeled WKYMVm binding on DC2.4 cells at the level comparable with that of unlabeled WKYMVm (Figure 1B). These results suggest that the DC2.4 cells express at least two different cell surface receptors for WKYMVm, and one of them is shared by WKYMVM and the other is shared by fMLF.
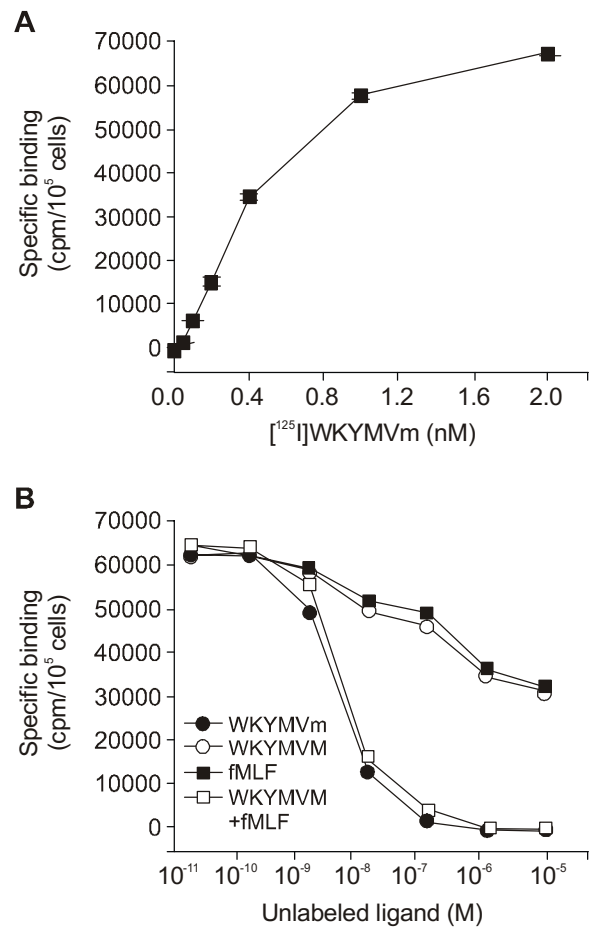
Figure 1. Expression of WKYMVm receptors in the DC2.4 cells. Several concentrations of [125I]-labeled WKYMVm was incubated with the DC2.4 cells for 3 h at $4^{o}C$ in the presence or absence of 10 µM of unlabeled WKYMVm (A). [125I]-labeled WKYMVm 2 nM was incubated with the DC2.4 cells for 3 h at $4^{o}C$ in the presence or several concentrations of unlabeled ligands, WKYMVm, WKYMVM, or fMLF (B) The level of the specific binding of [125I]-labeled WKYMVm was determined using a γ-ray counter. The results are presented as means ± S.E. of three independent experiments (A). In panel B, the data are representative of three independent experiments, which showed similar results.

DC2.4 cells express FPR1 and FPR2

WKYMVm was reported to bind FPR, FPRL1, and FPRL2 in human leukocytes (Le *et al.*, 1999; He *et al.*, 2000; Christophe *et al.*, 2001). The expression of mRNA for the different FPR family receptors in the DC2.4 cells was analyzed to identify different receptors by using semiquantitative RT-PCR analysis. As shown in Figure 2, the DC2.4 cells expressed both FPR1 and FPR2 but not the other FPR family. This correlates with the ligand binding analysis that showed the existence of multiple receptors for WKYMVm in the DC2.4 cells.

WKYMVM stimulates [Ca^{2+}]$_i$ increase in DC2.4 cells

Receptor function of both FPR1 and FPR2 expressed in the DC2.4 cells were examined by monitoring the increase in the [Ca^{2+}]$_i$ by specific ligand. When the DC2.4 cells were stimulated with several concentrations of WKYMVM, an increase in [Ca^{2+}]$_i$ was evoked in a concentration-dependent manner (Figure 3). The maximum activity was attained at 100 nM WKYMVM (Figure 3). 100 nM WKYMVM elicited flux of approximately 150 nM Ca^{2+} in the DC2.4 cells (Figure 3). FPR2 is reported to be linked to pertussis toxin (PTX)-sensitive G-protein coupled receptors (Tiffany *et al.*, 2001). Examination of the PTX effect on the WKYMVM-induced [Ca^{2+}]$_i$ increase in DC2.4 cells showed (Figure 3) that the cells preincubated with 100 ng/ml of PTX for 20 h caused the complete abolishment of WKYMVM induced [Ca^{2+}]$_i$ flux. This result suggests that the DC2.4 cells express the functional FPR2, and the receptor can be activated by WKYMVM in a PTX-sensitive manner. fMLF also induced the increase in [Ca^{2+}]$_i$ in a concentration-dependent manner showing PTX-sensitivity (data not shown). Since WKYMVM-induced [Ca^{2+}]$_i$ increase was more potent than fMLF-induced one (data not shown), in this study, we focused the effect of WKYMVM on the functional modulation of DC2.4 cells.
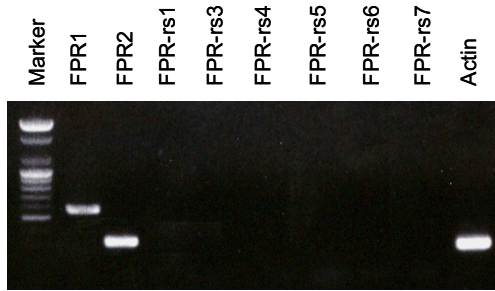

Figure 2. RT-PCR analysis of FPR family receptors in the DC2.4 cells. RT-PCR analysis was performed on the mRNA isolated from the cultured DC2.4 cells, as described in "Materials and Methods." The result presented is representative of three independent experiments.
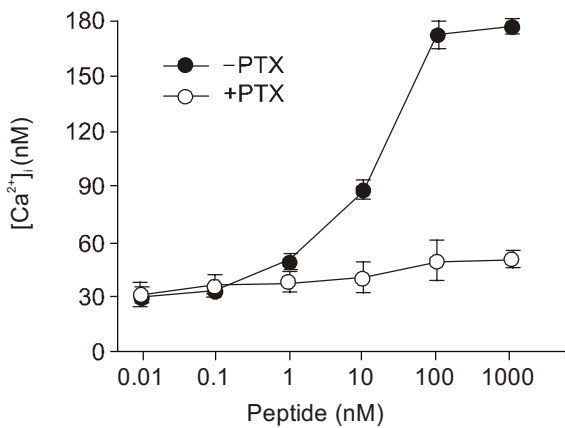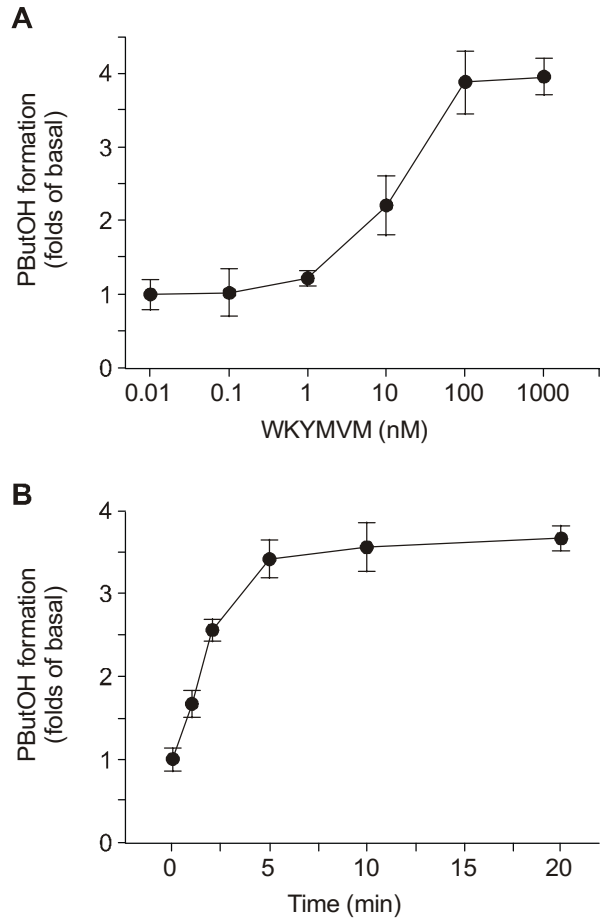

Figure 3. WKYMVM-induced cytosolic calcium increase in the DC2.4 cells. The DC2.4 cells were stimulated with various concentrations of WKYMVM in the presence or absence of PTX (100 ng/ml, 24 h), and the [Ca^{2+}]$_i$ was determined fluorometrically using fura-2/AM as described in "Materials and Methods". The peak [Ca^{2+}]$_i$ was monitored. The results are expressed as means ± S.E. of four independent experiments.

Figure 4. Activation of PLD by WKYMVM in the DC2.4 cells. The cultured DC2.4 cells were labeled with [^{3}H] myristic acid for 4 h at 37°C in RPMI 1640. After washing 3 times with PBS, the labeled cells were stimulated with various concentrations of WKYMVM in the presence of 0.5% n-butanol for 10 min (A) or with 1 µM of WKYMVM in the presence of 0.5% n-butanol for various time periods (B). PBt was resolved from the total lipids by thin layer chromatography. After chromatography, the PBt region was scrapped off and the radioactivity was counted. The data are presented as means ± S.E. from three separate experiments.

WKYMVM stimulates PLD activation *via* protein kinase C (PKC)- and Ca²⁺-dependent signaling in DC2.4 cells

PLD is regarded as a key enzyme that is involved in the modulation of several functions in leukocytic cells (Milella *et al.*, 1999; Bae *et al.*, 2000; Melendez *et al.*, 2001). Examination of WKYMVM effect on the PLD activation in the DC2.4 cells was carried out by using transphosphatidylation, a characteristic reaction of PLD in the presence of 0.5% normal butanol (n-butanol). WKYMVM stimulated the formation of PBt in a concentration dependent manner with maximum activity at 100 nM (Figure 4A). The kinetics of PBt formation with 1 μM WKYMVM showed that PBt formation was dramatically increased within 1 min

after stimulation and reached a plateau at 5 min (Figure 4B).

To investigate the signaling pathway leading to PLD activation by WKYMVM, the role of PKC in the process was examined. Preincubation of the DC2.4 cells with an effective concentration (5 μM) of the two PKC-inhibitors (Ro-31-8220 or GF109203X) prior to stimulation with WKYMVM blocked the PLD catalyzed PBt formation (Figure 5A). The peptide-stimulated PLD activity was almost completely inhibited when the DC2.4 cells were preincubated with 10 μM BAPTA/ AM prior to stimulation with WKYMVM (Figure 5A). However, pretreating the cells with PD98059 did not affect the PLD activity induced by WKYMVM (Figure 5A). Such results suggest that WKYMVM stimulates the PLD activation in a PKC- and Ca²⁺-dependent manner in the DC2.4 cells.
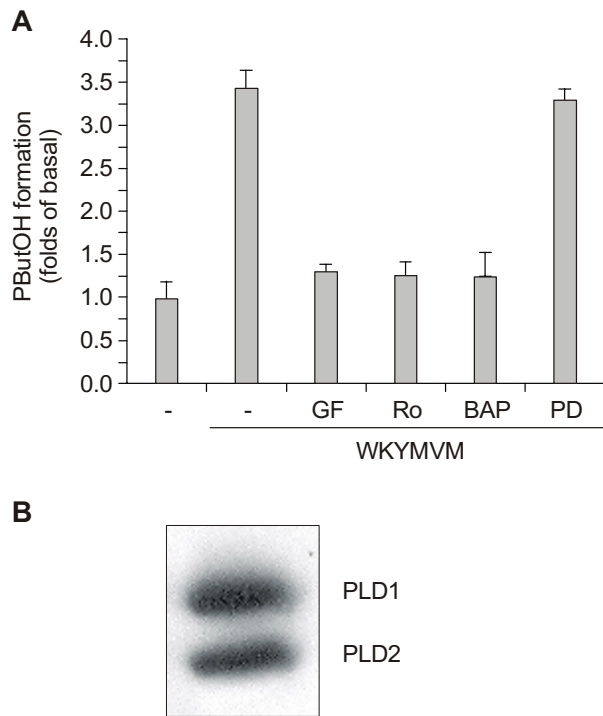
DC2.4 cells express PLD1 and PLD2

The identity of PLD isoform, which might be involved in the WKYMVM-dependent PBt formation in the DC 2.4 cells, was scrutinized using PLD antibodies: C-terminal-specific antibodies, as the immunological probes. The treated cell lysate was immunoprecipitated with Protein A Sepharose coupled to the C-terminal-specific antibodies that can react with both of the PLD1 and PLD2 (Lee *et al.*, 1997) and followed by immunoblotting analysis with the C-terminal PLD antibodies. Both PLD1 and PLD2 were identified in
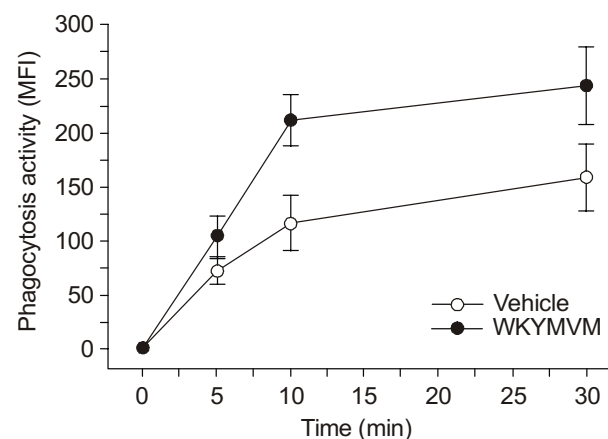
A

B

Figure 5. PKC-dependent PLD activation in the WKYMVM-treated DC2.4 cells and the immunological identification of PLD in the DC2.4 cells. The DC2.4 cells preloaded with [³H]myristic acid were preincubated with GF109203X (5 μM), Ro-31-8220 (5 μM), BAPTA/AM (10 μM), PD98059 (50 μM) or the vehicle only for 15 min at 37°C prior to stimulation with 1 μM WKYMVM in the presence of 0.5% n-butanol. The PBt was then separated from the total lipids by thin-layer chromatography. The Fuji BAS-2000 instrument was used to quantify each lipid. The data represent means ± S.E. from three separate experiments (A). The freshly cultured DC2.4 cells were lysed in a lysis buffer (20 mM Tris-HCl, pH 7.2, 10% glycerol, 150 mM NaCl, 1% Triton X-100,1% cholic acid, 1 mM Na₃VO₄, 50 mM NaF, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and 2 mg aliquots of the cell lysate were subjected to immunoprecipitation with protein A-Sepharose coupled to the C-terminal-specific antibodies followed by immunobloting with the anti-C-terminal-specific antibodies (B).

Figure 6. Enhancement of the DC phagocytosis by WKYMVM. DC2.4 cells (1×10⁵) were incubated with 1 mg/ml of FITC-dextran in 500 μl of PBS containing 1% human serum at 37°C in the presence or absence of 1 μM WKYMVM for several periods of time (0, 5, 10, and 30 min). After adding 2 ml of ice-cold PBS containing 1% human serum and 0.02% sodium azide, the samples were washed three times with ice-cold PBS and analyzed by using FACScan flow cytometer. The mean fluorescence intensity (MFI) was measured. The data are presented as means ± S.E. from three separate experiments.

the DC2.4 cells (Figure 5B).

WKYMVM stimulates phagocytic activity of DC2.4 cells

One of the important functions of iDCs is phagocytosis of pathogens or dying cells. Regulating the phagocytic activity is crucial for the efficient processing of the antigens and the presentation of the processed antigenic peptides by the MHC molecule (Guermonprez *et al.*, 2002; Granucci *et al.*, 2003). To assess the effect of FPR2 activation on the DC2.4 cells, the phagocytic activity was measured by using FITC-dextran. When FITC-dextran was added to the

DC2.4 cells, the cells spontaneously induced phagocytosis in a time-dependent manner (Figure 6). Stimulating the DC2.4 cells with 1 µM of WKYMVM significantly increased the phagocytic activity of the DC2.4 cells (Figure 6). This WKYMVM-induced enhancement of phagocytic activity was apparent within 5-30 min after adding the FITC-dextran (Figure 6).

WKYMVM-stimulated phagocytic activity is PLD-mediated

Several studies have reported that PA, a product of phosphatidylcholine hydrolysis by PLD, is involved in the modulating the leukocyte functions (Bae *et al.*, 1999; Giron-Calle and Froman, 2000; Kaldi *et al.*, 2002). In this study we tested whether or not PLD is involved in the WKYMVM-stimulated phagocytic activity in DC2.4 cells using n-butanol. In the transphosphatidyl reaction, a primary alcohol such as n-butanol acts as a PA acceptor resulting in the production phosphatidylalcohol instead of PA, while a non-primary alcohol such as tertiary butanol (*t*-butanol) does not, thereby allowing PA production (Bae *et al.*, 1999). WKYMVM stimulated the formation of PBt in the presence of 0.5% n-butanol in the DC2.4 cells, as shown in Figure 4A, while the PA formation during treatment with WKYMVM was not blocked in the presence of 0.5% *t*-butanol. Figure 7A shows that the WKYMVM-induced phagocytic activity of the DC2.4 cells was almost totally inhibited in the presence of 0.5% n-butanol but not in the presence of 0.5% *t*-butanol. These results suggest that PA production is essential for the phagocytic activity in DC2.4 cells. In order to support the hypothesis that PLD activation is essential for the phagocytic activity, we investigated the direct effect of PA on the phagocytosis of the DC2.4 cells. As shown in Figure 7B, the addition of PA in the DC2.4 cells caused an apparent increase in the phagocytic activity of the
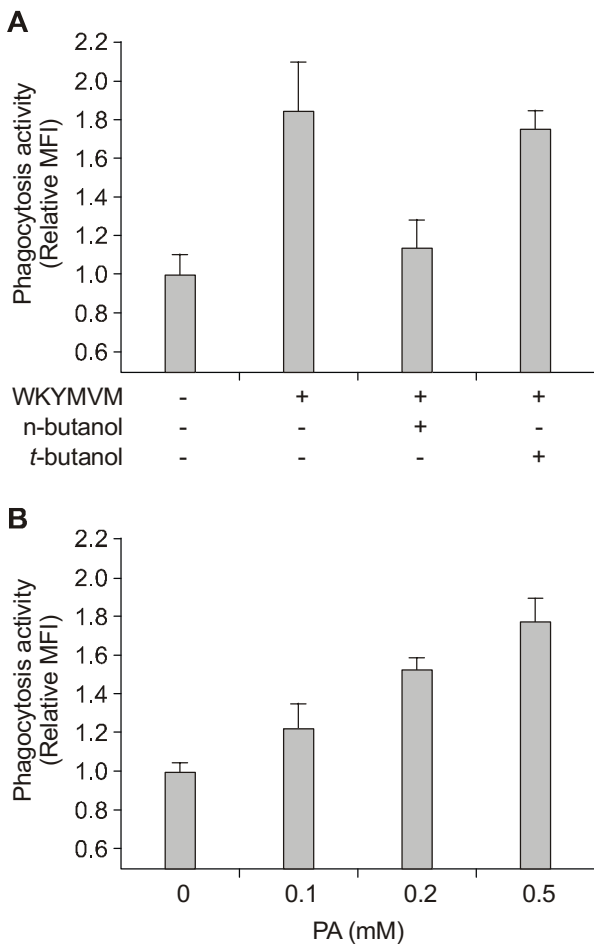

Figure 7. The role of PLD on the WKYMVM-induced DC phagocytosis. The phagocytic activity was measured as described in "Materials and Methods." The DC2.4 cells (1×10^5) were stimulated with 1 µM WKYMVM in the presence of 0.5% n-butanol or 0.5% t-butanol for 10 min at 37°C (A). The DC2.4 cells (1×10^5) were stimulated with various PA concentrations for 10 min at 37°C (B). The uptaken FITC-dextran by DC2.4 cells were analyzed using FACScan flow cytometry. The mean fluorescence intensity (MFI) was measured. The data are presented means ± S.E. from three separate experiments.
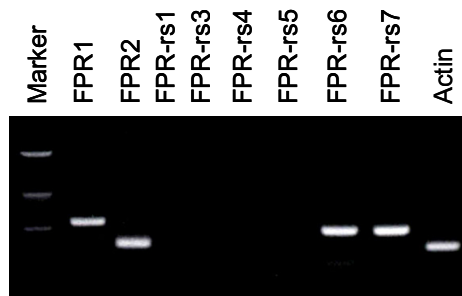

Figure 8. RT-PCR analysis of FPR family receptors in mouse bone marrow-derived DCs. RT-PCR analysis was performed on the mRNA isolated from the mouse bone marrow-derived DCs, as described in "Materials and Methods." The result presented is representative of three independent experiments.

cells. Therefore, the results suggest that WKYMVM stimulates the phagocytic activity of the DC2.4 cells *via* PLD activation.

Mouse bone marrow-derived DCs express FPR1 and FPR2

To support our notion that WKYMVM stimulates primary mouse DCs, we analyzed expression of mRNA for the different FPR family receptors in primary mouse bone marrow-derived DCs using semiquantitative RT-PCR analysis. As shown in Figure 8, primary mouse bone marrow-derived DCs expressed both FPR1 and FPR2. Unlike to the DC2.4 cells primary mouse DCs expressed two other FPR family receptors, FPR-rs6 and FPR-rs7.

Discussion

DCs have important role in immune responses against invading microbial pathogens, apoptotic cells and tumors. In order to perform these immunological functions, DCs have to uptake the antigens necessary for the initiation of immune responses. In this study we demonstrate that a synthetic chemoattractant peptide WKYMVM enhances phagocytic activity in mouse DCs, DC2.4. This result suggests that WKYMVM act as an important functional modulator of DCs. The investigation of the signaling leading to the enhancement of phagocytic activity in DC2.4 cells after peptide stimulation revealed that the peptide-induced phagocytic activity is PLD-dependent.

Although the phagocytic activity of DC is a crucial step for regulating the DC-mediated immune responses, the molecular mechanism involved in the process is not clearly understood. In this study we demonstrated that activating the phagocytosis of DC by WKYMVM is completely inhibited by the addition of n-butanol but not *t*-butanol. The result suggests that PLD activation by WKYMVM is essential for the phagocytic activity in the DCs. Since PA generation is the primary consequence of PLD activation (Lennartz, 1999; Cockcroft, 2001), investigation on the effect of the exogenous addition of PA on the phagocytic activity of DC2.4 cells was carried out. The phagocytic activity in the DC2.4 cells was indeed enhanced by the exogenously added PA (Figure 7B), supporting the hypothesis that PLD activation is necessary for the phagocytic activity in DC2.4 cells. Previously several reports suggested that the activation of PLD is essentially coupled to the phagocytosis in phagocytic cells, such as macrophages and neutrophils (Fallman *et al.*, 1992; Kusner *et al.*, 1996). In the process of phagocytosis of *Mycobacterium tuberculosis* or opsonized zymosan by macrophages, the inhibition of PLD by 2,3-diphosphoglycerate (a

PLD inhibitor) (Kanaho *et al.*, 1993) resulted in concentration-dependent reduction in phagocytosis (Kusner *et al.*, 1996). Furthermore, the phagocytosis of *M. tuberculosis* of 2,3-diphosphoglycerate treated macrophage was restored by addition of purified PLD (Kusner *et al.*, 1996). Fallman *et al.* demonstrated that the activation of PLD can act as an important signal regulating the engulfment of complement opsonized particles in the downstream of the signaling pathway of complement receptor type 1 and complement receptor type 3 in human neutrophils (Fallman *et al.*, 1993). Recently, Jackson and colleagues demonstrated that the PLD activation induced by Fcγ receptor regulates macrophage phagocytosis, by showing that phagocytosis of IgG-coated sheep red blood cells was inhibited by two inhibitors of PLD-mediated signaling, 2,3-diphosphoglycerate and n-butanol (Kusner *et al.*, 1999). The PA-activated protein kinases have been suggested to perform a role in the mechanism involved in regulating PA-mediated macrophage phagocytosis (Kusner *et al.*, 1999). We also observed that stimulating the DC2.4 cells with WKYMVM caused a dramatic increase in the tyrosine phosphorylation levels of several cellular proteins, including 120 kDa, 68 kDa, and 50 kDa (data not shown). The WKYMVM-induced tyrosine phosphorylation of the proteins was inhibited by the addition of 0.5% n-butanol but not 0.5% *t*-butanol (data not shown). The results suggest that several tyrosine kinases can be activated by WKYMVM *via* the PLD-mediated signaling pathway and potentially be involved in the regulation of DC phagocytosis.

On the cell surface receptors involved in the WKYMVM-induced phagocytic activity in mouse DCs, we demonstrated that DC2.4 mouse DCs express two FPR family receptors, FPR1 and FPR2 (Figure 2). Previously, Dahlgren and colleagues demonstrated that WKYMVM binds to FPRL1 and FPRL2 but not FPR (Christophe *et al.*, 2001). We also confirmed that primary mouse DCs express several different FPR family receptors using semiquantitative RT-PCR analysis (Figure 8). Taken together, the results suggest that WKYMVM may enhance the phagocytic activity of mouse DCs *via* activation of FPR family. Considering that the phagocytic activity by the DCs is an important initial step in the immune responses against the infected pathogens or tumor antigens, the identification of receptor which is crucial for the WKYMVM-induced phagocytosis in DCs will be important. Furthermore, the receptor will be regarded to be crucial target for modulating immune responses.

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