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Involvement of a phosphatidylinositol 3-kinase–p38 mitogen activated protein kinase pathway in antigen-induced IL-4 production in mast cells

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Abstract

We studied the involvement of phosphatidylinositol 3-kinase (PI3-kinase) in the antigen-induced IL-4 production in a rat mast cell line, RBL-2H3. The stimulation of IgE-sensitized RBL-2H3 cells by the antigen resulted in increased IL-4 mRNA levels followed by increased IL-4 production. Wortmannin and LY294002, PI3-kinase inhibitors, partially reduced both the antigen-induced increases in the IL-4 mRNA levels and IL-4 production in a concentration-dependent manner. Extracellular signal-regulated kinase, p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK), which belong to the MAPK family, were activated by the antigen stimulation, and the activation of p38 MAPK in addition to JNK was suppressed markedly by wortmannin. The phosphorylation of endogenous activating transcription factor-2, a substrate of p38 MAPK, was also inhibited by wortmannin. The specific p38 MAPK inhibitor SB203580 partially inhibited the antigen-induced IL-4 production at mRNA levels, but the MEK-1 inhibitor PD98059 enhanced it. These findings suggest that the activation of PI3-kinase and p38 MAPK is partially responsible for the antigen-induced IL-4 production in RBL-2H3 cells.

Keywords: IL-4; PI3-kinase; p38 MAP kinase; RBL-2H3 cells

1. Introduction

The antigen-induced aggregation of IgE binding to high-affinity IgE receptors (FcεRI) on mast cells induces degranulation, arachidonic acid release and production of a variety of cytokines including IL-4 [1]. IL-4 plays central roles in Th2-type immune responses, such as IgE production and immediate allergic inflammation [2,3] and might be involved in the exacerbation of allergic diseases.

Transcription of IL-4 gene has been studied in T-cells and mast cells. Stimulation of T-cell receptors induces the activation of two transcription factors; nuclear factor of activated T-cell (NF-AT) and activator protein-1 (AP-1). NF-AT and AP-1 bind cooperatively to their cognate binding sites in IL-4 promoter, and the binding of both NF-AT and AP-1 is necessary for maximum transcription in T-cells [4]. In addition, the involvement of several other transcription factors in IL-4 production by T-cells has

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also been suggested [5–7]. NF-AT is activated by the antigen stimulation in mast cells [8], but the regulation mechanism of IL-4 production in mast cells and T-cells differs from each other [9].

The aggregation of IgE/FcεRI with the antigen activates the tyrosine kinases, Lyn and Syk, followed by the phosphorylation of phospholipase Cγ and Vav [10,11]. The activated phospholipase Cγ generates inositol polyphosphate and diacylglycerol, which increases intracellular Ca^{2+} level and activates protein kinase C (PKC), respectively. The tyrosine phosphorylation of Vav results in the activation of small G-proteins, such as Ras and Rac [12,13]. The activation of these small G-proteins leads to the activation of MAPK superfamily; extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) [14,15]. The antigen stimulation of mast cells induces the activation of these MAPKs [16–18]. The antigen-induced degranulation of mast cells is highly dependent on both the activation of PKC and the increase in cytosolic Ca^{2+} concentration [19]. In contrast, the arachidonic acid release induced within a few minutes after the antigen stimulation is dependent on both the phosphorylation of cytosolic phospholipase A_2 by ERK and the increase in cytosolic Ca^{2+} concentration [16]. In addition, JNK is demonstrated to play crucial roles in the production of IL-2 [20] and TNF-α [21] by mast cells.

Phosphatidylinositol 3-kinase (PI3-kinase) is also activated by the antigen-stimulation in mast cells [22,23] and is implicated in various cellular responses, such as degranulation [22–24] and membrane ruffling [23,25]. The roles of PI3-kinase have been clarified by using the selective inhibitors of PI3-kinase, wortmannin [22] and LY294002 [26]. The TNF-α production by the mouse mast cell line MC/9 is inhibited by wortmannin [21]. PI3-kinase, which converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-triphosphate, regulates the activation of the small G-protein Rac [27], PKCξ [28], the serine/threonine kinase Akt [29], and MAPK family [18,21,30,31]. However, although significant roles of PI3-kinase in these signaling pathways have been described, the involvement of these pathways activated by PI3-kinase in the antigen-induced IL-4 production has not been elucidated. In this study, we examined the involvement of PI3-kinase and MAP kinases in the antigen-stimulated IL-4 production in rat mast cells.

2. Materials and methods

2.1. Cell treatment and measurement of IL-4

The rat mast cell line RBL-2H3 was kindly supplied by Dr. Michael A. Beaven (National Institutes of Health, Bethesda, MD, USA). RBL-2H3 cells (2×10^5 cells) were incubated overnight in 0.4 ml of Eagle's minimum essential medium (EMEM) containing 10% (v/v) fetal bovine serum (FBS, Flow Laboratories, North Ridge, NSW, Australia) and 0.1% (v/v) conditioned medium of the DNP-specific IgE-producing hybridoma (kindly supplied by Dr. Kazutaka Maeyama, Ehime University, Ehime, Japan) in each well of a 24-well cluster dish (Coster, Cambridge, MA, USA). After three washes with EMEM, the cells were incubated in 0.2 ml of EMEM containing 5% FBS in the presence or absence of wortmannin (Biomol, Plymouth Meeting, PA, USA), LY294002 (Biomol) SB203580 (Calbiochem-Novabiochem, San Diego, CA, USA), or PD98059 (New England Biolabs, Beverly, MA, USA) for 10 min. The cells were then stimulated for the periods indicated by the antigen DNP-HSA (50 ng/ml) (Sigma, St. Louis, MO, USA). The conditioned medium was collected, centrifuged at $220\times g$ and 4°C for 5 min. The IL-4 levels in the supernatant fraction were determined using a rat IL-4 ELISA kit (BioSource, Camarillo, CA, USA). In some cases, after the antigen stimulation, the cells were washed with ice-cold EMEM, and then sonicated in 0.2 ml of EMEM, and the IL-4 levels in the cell lysates were determined as described above.

2.2. Semiquantitation of IL-4 mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

RBL-2H3 cells (1×10^6 cells/well) in a 6-well cluster dish (Coster) were incubated for various periods in EMEM containing 5% FBS. After the antigen stimulation as described above, total RNA was prepared from each sample by acid guanidinium-phenol-chloroform extraction, and the yield of RNA ex-

tracted was determined by spectrophotometry. One microgram of RNA from each sample was reverse transcribed in 20 μl of the buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) containing 5 μM of random hexamer oligonucleotides (Gibco-BRL, Gaithersberg, MD, USA), 200 U of the reverse transcriptase from moloney murine leukemia virus (Gibco-BRL), 0.5 mM deoxyribonucleoside triphosphates (dNTP, Pharmacia Biotech, Uppsala, Sweden) and 10 mM dithiothreitol. The PCR primers for rat IL-4 were: (former) 5′-ACCTTGCTGTC-ACCCTGTTCTTGC-3′ and (reverse) 5′-GTTGTG-AGCGTGGACTCATTC-3′, which amplify a 352-base pair (bp) IL-4 DNA fragment. PCR was performed for 25 cycles; 1 min denaturation at 94°C, 1 min annealing at 58°C, and 2 min extension at 72°C, using a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer Cetus, Norwalk, CT, USA). The rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (a housekeeping gene) was used as an internal control as described before [32]. In such conditions for RT-PCR, levels of each mRNA could be determined semiquantitatively. The levels of mRNA for IL-4 and GAPDH were quantified by scanning densitometry, and the ratio of the IL-4 mRNA density to the GAPDH mRNA density in each point was calculated.

2.3. In vitro assay for p38 MAPK

RBL-2H3 cells (1.0×10^6 cells/well) in a 6-well cluster dish were washed with PIPES-buffer (25 mM PIPES, pH 7.2, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$ and 1 mM $CaCl_2$), and stimulated by the antigen DNP-HSA (50 ng/ml) in 1.0 ml of PIPES buffer. After stimulation of the cells for the periods indicated, the cells were lysed in 0.5 ml of lysis buffer (20 mM HEPES, pH 7.3, 1% Triton X-100, 10% glycerol, 50 mM NaF, 2.5 mM *p*-nitrophenylphosphate, 10 μg/ml PMSF, 1 mM Na_3VO_4, 5 μg/ml leupeptin and 1 mM dithiothreitol). The cell lysate was centrifuged at $18\,600\times g$ and 4°C for 20 min. To the supernatant fraction, 3 μg anti-p38 MAPK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 20 μl protein A-Sepharose slurry (Pharmacia Biotech) were added and incubated for 3 h at 4°C. The immunoprecipitate of p38 MAPK was washed with washing buffer (10 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 150 mM NaCl) and then washed with kinase assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) three times each. The kinase reaction was initiated by adding 20 μl kinase buffer containing 500 ng ATF-2 (amino acids 1–505) (Santa Cruz Biotechnology), 10 μM ATP and 74 kBq [γ-^{32}P]ATP (111 TBq/mmol, DuPont/NEN, Boston, MA, USA). After incubation for 30 min at 30°C, the reactions were centrifuged at $18\,600\times g$ and 4°C for 5 min, and ATF-2 in the supernatant fraction was separated on a 10% SDS-PAGE gel. Phosphorylated ATF-2 was visualized by Bioimage analyzer (GS-250, Bio-Rad, Melville, NY, USA) and by autoradiography.

2.4. Immunoblotting of p38 MAPK, phospho-p38 MAPK, phospho-JNK, JNK and ERK

After the antigen stimulation, the cells (5×10^5 cells/well) in a 12-well cluster dish (Coster) were lysed in 0.075 ml of lysis buffer. Proteins in the cell lysate were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). p38 MAPK, phospho-p38 MAPK, phospho-JNK, JNK and ERK were immnoblotted using polyclonal antibodies against the epitope corresponding to amino acids 341–360 mapping at the carboxy terminus of p38 MAPK (Santa Cruz Biotechnology), p38 MAPK phosphorylated at Thr-180 and Tyr-182 (New England Biolabs), JNK phosphorylated at Thr-183 and Tyr-185 (Promega, Madison, WI, USA), p54 JNK-2 (Santa Cruz Biotechnology), and the epitope corresponding to amino acids 333–367 mapping at the carboxy terminus of p43 ERK (Upstate Biotechnology, Lake Placid, NY, USA), respectively.

2.5. Immunoblotting of phospho-ATF-2 in nuclei

RBL-2H3 cells (1.0×10^6 cells/well) in a 6-well cluster dish were stimulated by the antigen DNP-HSA as described above. After incubation for the periods indicated, the cells were harvested in ice-cold phosphate-buffered saline (pH 7.4). The cells were incubated in 0.1 ml of Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 1 mM PMSF) for 15 min on ice. Nonidet P-40 (Sigma) was then added to make a

final concentration at 0.25%. The mixture was vortexed vigorously for 10 s and left on ice for 1 min. Nuclei were pelleted by centrifugation at $3000 \times g$ and 4°C for 5 min, and proteins in the pelleted nuclei were extracted with 0.1 ml of Buffer B (20 mM HEPES, pH 7.9, 400 mM KCl, 0.1 mM EDTA 1 mM DTT, and 1 mM PMSF) on ice for 30 min. After centrifugation at $18\,600 \times g$ and 4°C for 15 min, the supernatant fractions were used as nuclear extracts. ATF-2 and phosphorylated ATF-2 in nuclear extracts were immunoblotted using polyclonal antibodies against the epitope corresponding to amino acids 1–96 mapping at the amino terminus of human ATF-2 (Santa Cruz, Biotechnology) and against ATF-2 phosphorylated at Thr-71 (New England Biolabs), respectively.

2.6. Statistical analysis

Results are expressed as means ± S.E.M. of four culture dishes in one representative experiment of at least three independent experiments. Comparisons of results were made by Student's unpaired *t*-test.

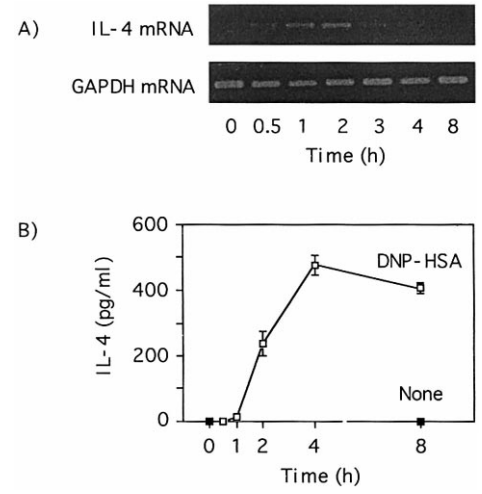

Fig. 1. Time course of IL-4 mRNA levels and IL-4 production in the antigen-stimulated RBL-2H3 cells. IgE-sensitized RBL-2H3 cells (1×10^6 cells (A) or 2×10^5 cells (B)) were stimulated with DNP-HSA (50 ng/ml) for the periods indicated. (A) Total RNA was extracted and the levels of mRNA for IL-4 and GAPDH were detected by RT-PCR as described in Section 2.2. (B) The concentrations of IL-4 in the conditioned medium were determined by ELISA. Closed squares indicate IL-4 concentrations in the conditioned medium of unstimulated cells. Vertical bars represent S.E.M. from four wells. The results were confirmed by two additional independent experiments.

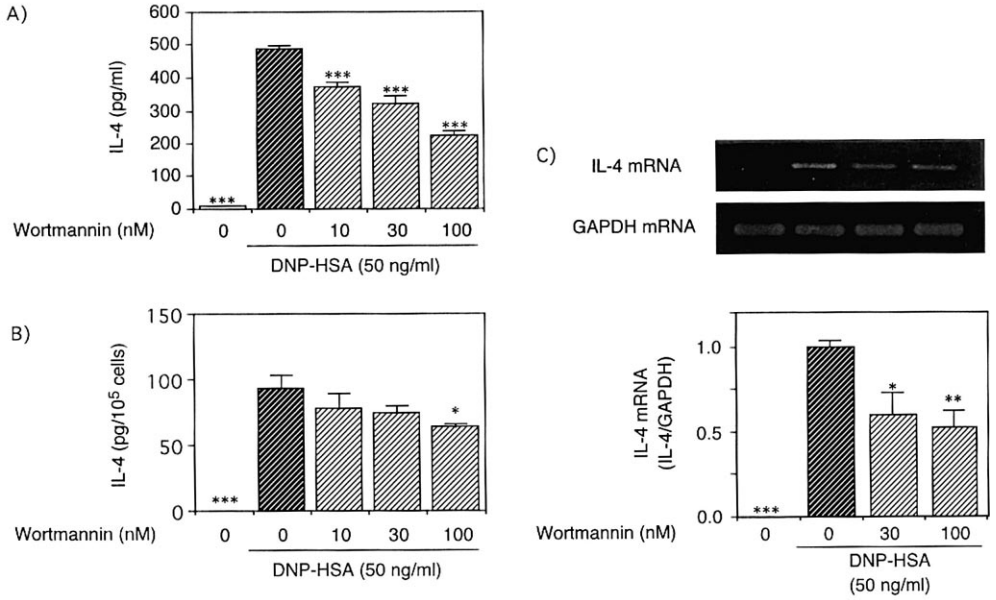

Fig. 2. Effects of wortmannin on the antigen-induced IL-4 production. IgE-sensitized RBL-2H3 cells (2×10^5 cells (A,B) or 1×10^6 cells (C)) were treated for 10 min in the presence of the indicated concentrations of wortmannin, then stimulated with DNP-HSA (50 ng/ml) in the continued presence of wortmannin. Concentrations of IL-4 in the conditioned medium (A) and in the cells (B) at 4 h were determined by ELISA. Vertical bars represent S.E.M. from four wells. (C) Two hours after the antigen stimulation, total RNA was extracted and the levels of mRNA for IL-4 and GAPDH were detected by RT-PCR as described in Section 2.2. The ratio of IL-4 mRNA density to GAPDH mRNA density was determined in three independent experiments and indicated in the lower panel. The density ratio of the antigen-stimulated control is set to 1.0. Statistical significance; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs. the antigen-stimulated control.

3. Results

3.1. IL-4 production induced by the antigen in RBL-2H3 cells

IgE-sensitized RBL-2H3 cells were stimulated by the antigen for the periods indicated, and then the IL-4 levels in the conditioned medium and the IL-4 mRNA levels were determined. As shown in Fig. 1B, IL-4 was detected at 2 h and attained the maximum level 4 h after the antigen stimulation. IL-4 mRNA was not detected in the non-stimulated cells, whereas the antigen stimulation markedly increased the IL-4 mRNA level with a maximum at 2 h (Fig. 1A).

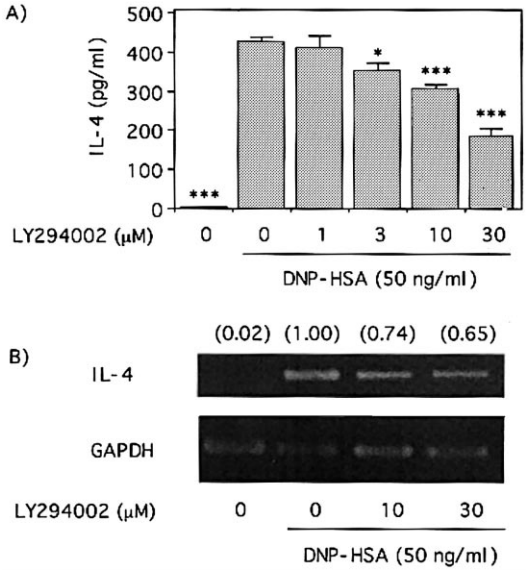

Fig. 3. Effects of LY294002 on the antigen-induced IL-4 production. IgE-sensitized RBL-2H3 cells (2×10^5 cells (A) or 1×10^6 cells (B)) were treated for 10 min in the presence of the indicated concentrations of LY294002, then stimulated with DNP-HSA (50 ng/ml) in the continued presence of LY294002. (A) Concentrations of IL-4 in the conditioned medium at 4 h were determined by ELISA. Vertical bars represent S.E.M. from four wells. Statistical significance; $^*P<0.05$ and $^{***}P<0.001$ vs. the antigen-stimulated control. (B) Two hours after the antigen stimulation, total RNA was extracted and the levels of mRNA for IL-4 and GAPDH were detected by RT-PCR as described in Section 2.2. The numbers in parentheses in B indicate the ratio of IL-4 mRNA density to GAPDH mRNA density. The density ratio of the antigen-stimulated control is set to 1.0.

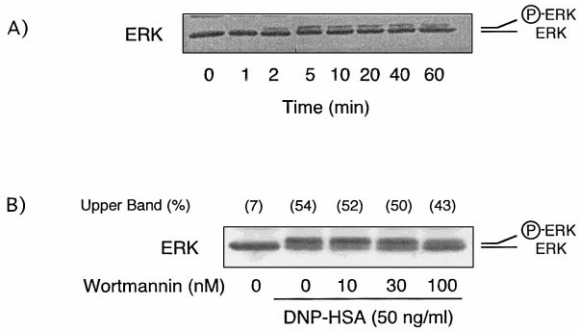

Fig. 4. Antigen-induced activation of ERK and effects of wortmannin. (A) IgE-sensitized RBL-2H3 cells (5×10^5 cells) were stimulated with DNP-HSA (50 ng/ml) for the periods indicated. (B) The cells were preincubated for 10 min in the presence of the indicated concentrations of wortmannin, then stimulated with DNP-HSA (50 ng/ml) for 2 min in the continued presence of wortmannin. Proteins in the cell lysate were separated by electrophoresis on an 8% SDS-PAGE gel and ERK was immunoblotted. Phosphorylated ERK (Ⓟ-ERK) and unphosphorylated ERK (ERK) are indicated. The numbers in parentheses in B indicate the proportion of the phosphorylated ERK as determined by densitometric analysis. The results were confirmed by two additional independent experiments.

3.2. Effects of the PI3-kinase inhibitor wortmannin and LY294002 on the antigen-induced IL-4 production

Pretreatment for 10 min with wortmannin decreased the IL-4 levels in the conditioned medium 4 h after the antigen stimulation in a concentration-dependent manner (Fig. 2A). To clarify whether wortmannin inhibits IL-4 production or its release to medium from inside the cells, IL-4 levels in the cells and IL-4 mRNA levels 2 h after the antigen stimulation were determined. As shown in Fig. 2B,C, wortmannin reduced both the IL-4 levels in the cells and the IL-4 mRNA levels. Another PI3-kinase inhibitor LY294002 also reduced the IL-4 levels in the conditioned medium (Fig. 3A) and IL-4 mRNA levels (Fig. 3B) in a concentration-dependent manner.

3.3. Effects of wortmannin on the activation of ERK, p38 MAPK and JNK

The activation of ERK was determined by the mobility shift of ERK in SDS-PAGE. The mobility shift was induced 2 min after the antigen stimulation and continued for at least 60 min (Fig. 4A). Pretreat-

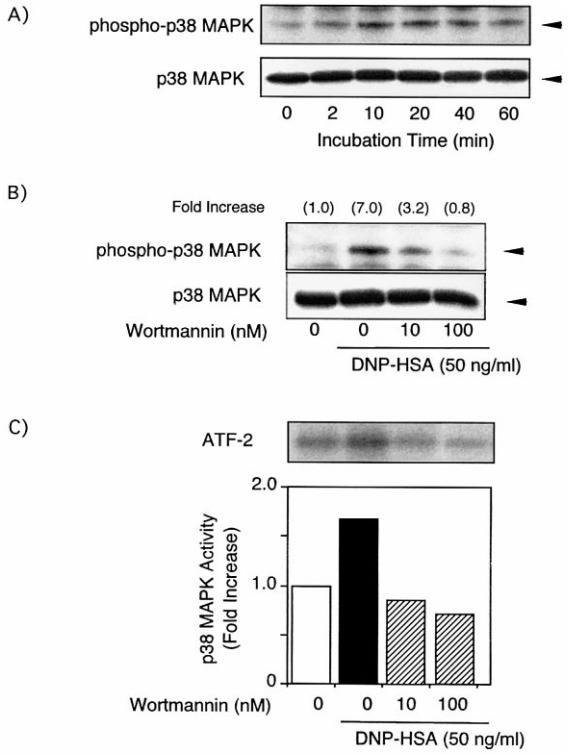

Fig. 5. Antigen-induced activation of p38 MAPK and effects of wortmannin. (A) IgE-sensitized RBL-2H3 cells (5×10^5 cells) were stimulated with DNP-HSA (50 ng/ml) for the periods indicated. (B) IgE-sensitized RBL-2H3 cells (5×10^5 cells) were preincubated for 10 min in the presence of the indicated concentrations of wortmannin, then stimulated with DNP-HSA (50 ng/ml) for 20 min in the continued presence of wortmannin. Proteins in the cell lysate were separated by electrophoresis on an 8% SDS-PAGE gel and p38 MAPK and phospho-p38 MAPK were immunoblotted. The numbers in parentheses in B indicate the fold increase in phospho-p38 MAPK as determined by densitometric analysis. The value of the unstimulated control is set to 1.0. (C) IgE-sensitized RBL-2H3 cells (1×10^6 cells) were treated as described in B, and the p38 MAPK activity in the cell lysate was determined as described in Section 2.2. The phosphorylation of ATF-2 was analyzed by autoradiography and a Bioimage analyzer. The value of the unstimulated control is set to 1.0. The results were confirmed by two additional independent experiments.

ment with wortmannin at 100 nM resulted in partial reduction of the percentage of upper band (phosphorylated ERK) at 2 min, but the reduction by 10 and 30 nM wortmannin was very slight (Fig. 4B).

After the antigen stimulation, phosphorylation of p38 MAPK increased from 10 to 40 min, but p38 MAPK protein content did not change during the period examined (Fig. 5A). The increases in the phosphorylation (Fig. 5B) and the kinase activity (Fig. 5C) of p38 MAPK 20 min after the antigen stimulation were markedly inhibited by the pretreatment with wortmannin at 10 and 100 nM.

The antigen stimulation induced the phosphorylation of JNKs, p46 JNK (JNK1) and p54 JNK (JNK2). The increase in the phosphorylation of JNKs 40 min after the antigen stimulation was reduced by the pretreatment with wortmannin at 10 and 100 nM, although the protein contents of JNK2 were not changed (Fig. 6).

3.4. Effects of wortmannin on the antigen-induced ATF-2 phosphorylation

As an index of p38 MAPK activity in the antigen-stimulated cells, the phosphorylation of endogenous ATF-2 was determined. The phosphorylation of ATF-2 increased time-dependently until 20 min after the antigen challenge and then declined, without changes in ATF-2 protein contents (Fig. 7A). The phosphorylation of ATF-2 at 20 min was inhibited by wortmannin in a concentration-dependent manner (Fig. 7B).

3.5. Effects of the MEK-1 inhibitor PD98059 on the antigen-induced ERK activation and IL-4 production

PD98059 was used to examine the role of ERK in

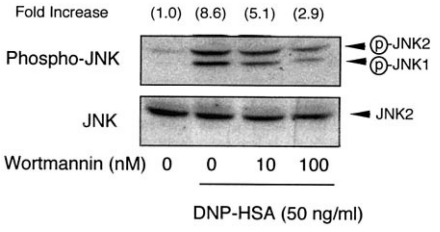

Fig. 6. Effects of wortmannin on the antigen-induced phosphorylation of JNK. IgE-sensitized RBL-2H3 cells (5×10^5 cells) were preincubated for 10 min in the presence of the indicated concentrations of wortmannin, then stimulated with DNP-HSA (50 ng/ml) for 40 min in the continued presence of wortmannin. Proteins in the cell lysate were separated by electrophoresis on an 8% SDS-PAGE gel and phospho-JNK was immunoblotted. Then, JNK on the same membrane was reblotted. Phosphorylated JNK ((P)-JNK1 and (P)-JNK2) and JNK2 are indicated. The numbers in parentheses indicate the fold increase in the phosphorylated JNK as determined by densitometric analysis. The results were confirmed by two additional independent experiments.

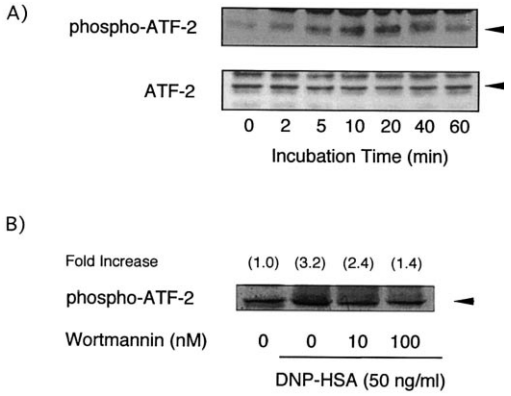

Fig. 7. Antigen-induced phosphorylation of endogenous ATF-2 and effects of wortmannin. (A) IgE-sensitized RBL-2H3 cells (1.0×10^6) were stimulated with DNP-HSA (50 ng/ml) for the periods indicated. (B) The cells were preincubated for 10 min in the presence of the indicated concentrations of wortmannin, then stimulated with DNP-HSA (50 ng/ml) for 20 min in the continued presence of wortmannin. Proteins in the nuclear extracts were separated by electrophoresis on an 8% SDS-PAGE gel, and ATF-2 and phosphorylated ATF-2 (phospho-ATF-2) were immunoblotted. The numbers in parentheses in B indicate the fold increase in phospho-ATF-2 as determined by densitometric analysis. The value of the unstimulated control is set to 1.0. The results were confirmed by two additional independent experiments.

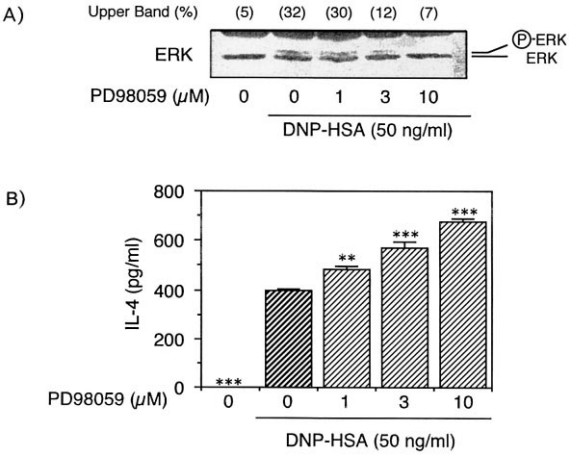

Fig. 8. Effects of PD98059 on the antigen-induced ERK activation and IL-4 production. IgE-sensitized RBL-2H3 cells (5×10^5 cells (A) or 2×10^5 cells (B)) were preincubated for 10 min in the presence of PD98059 at the indicated concentrations, then stimulated with DNP-HSA (50 ng/ml) for 2 min (A) or 4 h (B) in the continued presence of PD98059. (A) ERK activation was determined as described in the legend to Fig. 3. The numbers in parentheses indicate the fold increase in the phosphorylated ERK (Ⓟ-ERK) as determined by densitometric analysis. (B) Concentrations of IL-4 in the conditioned medium at 4 h was determined by ELISA. Vertical bars represent S.E.M. from four wells. The results were confirmed by two additional independent experiments. Statistical significance; $^{**}P<0.01$ and $^{***}P<0.001$ vs. the antigen-stimulated control.

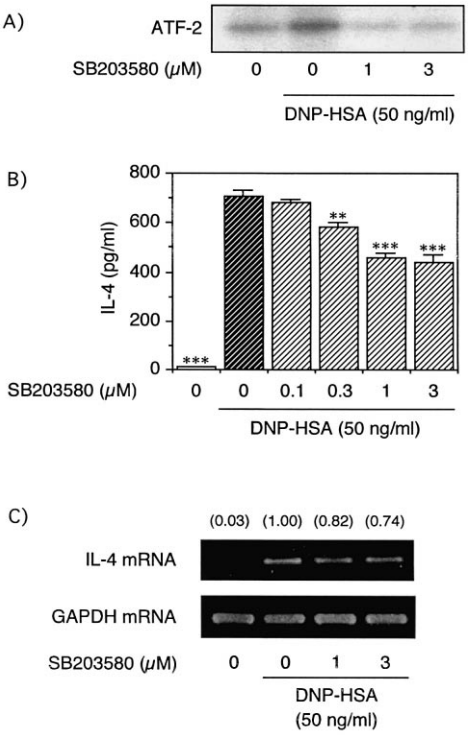

Fig. 9. Effects of SB203580 on the antigen-induced activation of p38 MAPK and IL-4 production. (A) IgE-sensitized RBL-2H3 cells (1×10^6 cells) were stimulated with DNP-HSA (50 ng/ml) for 20 min. Kinase activities in the anti-p38 MAPK immunoprecipitates from the cell lysates were determined in the presence of the indicated concentrations of SB203580. (B) IgE-sensitized cells (2×10^5 cells) were preincubated for 10 min in the presence of the indicated concentrations of SB203580, then stimulated for 4 h with DNP-HSA (50 ng/ml) in the continued presence of SB203580. Concentrations of IL-4 in the conditioned medium were determined by ELISA. Vertical bars represent S.E.M. from four wells. The results were confirmed by two additional independent experiments. (C) Two hours after the addition of the antigen, total RNA was extracted and levels of mRNA for IL-4 and GAPDH were detected by RT-PCR as described in Section 2.2. The numbers in parentheses in C indicate the ratio of IL-4 mRNA density to GAPDH mRNA density. The density ratio of the antigen-stimulated control is set to 1.0. The results were confirmed by two additional independent experiments.

IL-4 production. Pretreatment with PD98059 for 10 min inhibited the antigen-induced mobility shift of ERK at 2 min in a concentration-dependent manner (Fig. 8A). PD98059 at these concentrations enhanced the antigen-induced IL-4 production (Fig. 8B).

3.6. Effect of the p38 MAPK inhibitor SB203580 on IL-4 production

SB203580 at 1 and 3 μM completely inhibited p38 MAPK activity in vitro (Fig. 9A). However, as shown in Fig. 9B, SB203580 at concentrations up to 3 μM showed partial inhibition of the antigen-induced IL-4 production at 4 h. The effect was a concentration-dependent, and maximum inhibition was attained at 1 μM SB203580. The antigen-induced increase in the level of IL-4 mRNA at 2 h was also partially inhibited by 1 and 3 μM SB203580 (Fig. 9C).

4. Discussion

The findings of the present study indicated that PI3-kinase–p38 MAPK pathway is partially, but significantly, involved in the antigen-induced IL-4 production by rat mast cells. The antigen-stimulated mast cells produce several cytokines, such as IL-4 [1], IL-6 [33] and TNF-α [34]. The PI3-kinase inhibitor wortmannin does not inhibit IL-6 production [33], but partially inhibits production of IL-4 (this paper) and TNF-α [21]. In contrast with IL-4 production, the antigen-induced TNF-α production is not inhibited by the p38 MAPK inhibitor, but is regulated by a MEKK-JNK pathway which is also inhibited by wortmannin [22]. Thus, the production of each cytokine by the antigen-stimulated mast cells is regulated in a different way.

PI3-kinase plays important roles not only in the degranulation of mast cells [22–24,33], but also in the vesicle transport [35]. Although IL-4 release is dependent on the vesicle transport and/or the degranulation, it is unlikely that the release of IL-4 from mast cells is inhibited by wortmannin, because wortmannin reduced the levels of IL-4 both in the cells and in the conditioned medium (Fig. 2A,B). In addition, wortmannin-induced reduction of IL-4 mRNA levels (Fig. 2C) paralleled the reduction of IL-4 levels in the conditioned medium (Fig. 2A), indicating that wortmannin partially inhibits IL-4 production at mRNA level. Although wortmannin inhibits other enzymes, such as myosin light chain kinase [22], inhibition of IL-4 production by wortmannin seemed to be due to the inhibition of PI3-kinase, because the other inhibitor of PI3-kinase, LY294002 [26], also inhibited the antigen-induced IL-4 production (Fig. 3). In addition, SB203580, an inhibitor of p38 MAPK, partially inhibited IL-4 production (Fig. 9B) in parallel with the reduction of IL-4 mRNA levels (Fig. 9C), indicating that SB203580 also inhibits IL-4 production at mRNA level. These findings indicate that PI3-kinase and p38 MAPK regulate the transcription of IL-4 gene. However, it remains to be elucidated whether wortmannin and SB203580 reduce the stability of IL-4 mRNA, and whether they inhibit the translation of IL-4.

PI3-kinase regulates the activation of small G-proteins, such as Rac [12,13], which activates p21-activated kinase [14,36] resulting in the activation of a p38 MAPK pathway. The activation of Rac also leads to the activation of JNK pathway [15]. Wortmannin inhibits both JNK phosphorylation (Fig. 6) and p38 MAPK activation (Fig. 5) indicating that wortmannin inhibits these MAP kinase activation via the inhibition of the Rac activation. In a cell-free system, it is reported that the Rac activation by tyrosine-phosphorylated Vav is inhibited by the substrates of PI3-kinase, but is enhanced by the products of PI3-kinase [37]. In addition, Bruton's tyrosine kinase is involved in antigen-induced activation of JNK in mast cells [38] and wortmannin and LY294002 inhibit the Bruton's tyrosine kinase-dependent activation of JNK [39]. Further investigation is necessary to clarify whether Vav and Bruton's tyrosine kinase also regulate in the activation of p38 MAPK in the antigen-stimulated mast cells.

In the present study, wortmannin inhibited JNK phosphorylation (Fig. 6) consistent with our previous finding that wortmannin inhibits the antigen-induced JNK activation [16]. Inhibition by wortmannin of JNK activation was less than that of p38 MAPK. Our finding does not coincide with the report by Ishizuka et al. [21] indicating that, in mouse mast cell line MC/9, wortmannin completely inhibits the antigen-induced JNK activation, but partially inhibits p38 MAPK activation. Therefore, it is possible that the signaling pathway for the activation of p38 MAPK and JNK is different between RBL-2H3 cells and MC/9 cells. In contrast, wortmannin did not inhibit the activation of ERK in both RBL-2H3 cells (Fig. 4) and MC/9 cells [21]. Thus, PI3-kinase regu-

lates the activation of p38 MAPK and JNK, but not ERK in mast cells, and the inhibitors of PI3-kinase might be useful to clarify roles of p38 MAPK and JNK in mast cells.

IL-4 transcription in mast cells and T-cells is mainly regulated by NF-AT and AP-1 [4,9]. The activation of NF-AT alone induces IL-4 production, and AP-1 enhances the NF-AT-induced responses [4]. Although PI3-kinase is suggested to be involved in NF-AT activation, wortmannin does not inhibit NF-AT activation in T-cells [40]. Therefore, wortmannin might inhibit, through inhibiting the activation of p38 MAPK and JNK, the activation of AP-1, but not NF-AT, in the antigen-stimulated mast cells resulting in the partial inhibition of IL-4 production.

The precise mechanism by which p38 MAPK regulates IL-4 transcription remains to be elucidated. Although ATF-2, which forms heterodimer with cAMP responsive element (CRE) binding protein family members or AP-1 components, binds to CRE, CRE is not found in the IL-4 promoter region [5–7]. p38 MAPK also phosphorylates another transcription factor, myocyte-enhancer factor 2C, which is involved in the induction of c-Jun expression [41]. Because the antigen stimulation induces *c-fos* and *c-jun* expression [42], p38 MAPK might play a role in the induction of AP-1 components.

In contrast with p38 MAPK, the antigen-induced activation of ERK was not inhibited by wortmannin (Fig. 4). However, our finding that PD98059, a MEK-1 inhibitor, enhanced the antigen-stimulated IL-4 production (Fig. 8) suggests that ERK negatively regulates the antigen-induced IL-4 production. ERK induces the MAPK phosphatase MKP-3 [43] which deactivates MAPKs. In addition, inhibition of ERK by PD98059 activates p38 MAPK in HeLa cells [44]. Thus, ERK might regulate activation of p38 MAPK and JNK. In addition, because PD98059 enhances the antigen-induced expression of a reporter construct under the control of TNF-α promoter [21], ERK might have some unidentified regulatory roles in gene expression of certain cytokines.

In conclusion, PI3-kinase regulates the antigen-induced activation of p38 MAPK and JNK, and the PI3-kinase/p38 MAPK pathway is involved, in part, in the antigen-induced IL-4 production in RBL-2H3 cells.

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