Characterization of a Mouse Second Leukotriene B4 Receptor, mBLT2

BLT2-DEPENDENT ERK ACTIVATION AND CELL MIGRATION OF PRIMARY MOUSE KERATINOCYTES*

Received for publication, November 24, 2004, and in revised form, April 22, 2005
Published, JBC Papers in Press, May 2, 2005, DOI 10.1074/jbc.M413257200

Yoshiko Iizuka‡, Takehiko Yokomizo‡‡, Kan Terawaki‡, Mayumi Komine¶, Kunihiko Tamaki‡, and Takao Shimizu‡
From the Departments of Biochemistry and Molecular Biology and Dermatology, Faculty of Medicine, The University of Tokyo and PRESTO of Japan Science and Technology Agency, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

Leukotriene B4 (LTB4) is a potent chemoattractant and activator for granulocytes and macrophages and is considered to be an inflammatory mediator. Two G-protein-coupled receptors for LTB4, BLT1 and BLT2, have been cloned from human and shown to be high and low affinity LTB4 receptors, respectively. To reveal the biological roles of BLT2 using mouse disease models, we cloned and characterized mouse BLT2. Chinese hamster ovary cells stably expressing mouse BLT2 exhibited specific binding to LTB4, LTB4-induced calcium mobilization, inhibition of adenyl cyclase, and phosphorylation of extracellular signal-regulated kinase. We found that Compound A (4’-[(pentanoyl (phenyl) amino) methyl]-1, 1-biphenyl-2-carboxylic acid) was a BLT2-selective agonist and induced Ca2+ mobilization and phosphorylation of extracellular signal-regulated kinase through BLT2, whereas it had no effect on BLT1. 12-epi LTB4 exhibited a partial agonistic activity against mBLT1 and mBLT2, whereas 6-trans-12-epi LTB4 did not. Northern blot analysis showed that mouse BLT2 is expressed highly in small intestine and skin in contrast to the ubiquitous expression of human BLT2. By in situ hybridization and the reverse transcriptase polymerase chain reaction, we demonstrated that mouse BLT2 is expressed in follicular and interfollicular keratinocytes. Compound A, LTB4, and 12-epi LTB4 all induced phosphorylation of extracellular signal-regulated kinase in primary mouse keratinocytes. Furthermore, Compound A and LTB4 induced chemotaxis in primary mouse keratinocytes. These data suggest the presence of functional BLT2 in primary keratinocytes.

Leukotriene B4 (LTB4): 5(S), 12[R]-dihydroxy-6, 14-cis-8, 10-trans-eicosatetraenoic acid (1) is biosynthesized from arachidonic acid released from membrane phospholipids by the action of cytosolic and various types of phospholipase A2s (2). Two enzymes, 5-lipoxygenase (3, 4) and LTA4 hydrolase (5, 6), are required for LTB4 biosynthesis from arachidonic acid (7, 8). LTB4 is a potent proinflammatory mediator mainly synthetized by phagocytic cells, principally polymorphonuclear leukocyte and macrophage, which responds to infectious and inflammatory stimuli. LTB4 induces granulocyte migration, degranulation, superoxide generation, and adhesion to vascular endothelial cells. It plays an important role in the initial phase of the inflammatory response and is thought to be involved in a number of inflammatory diseases including psoriasis (9), bronchial asthma (10), rheumatoid arthritis (11) inflammatory bowel diseases (12), and ischemic tissue damage (13). Recently, LTB4 was shown to act as an immunomodulator by recruiting CD4+ early effector and CD8+ cytotoxic effector T-cells to inflamed tissues (14–16), suggesting novel roles of LTB4 in immunological disorders. Thus, many BLT antagonists have been currently under development as novel anti-inflammatory and anti-allergic drugs.

Two types of LTB4 receptors (BLT1 and BLT2) have been isolated. BLT1 (17) and BLT2 (18–20) are high and low affinity LTB4 receptors, respectively. These receptors belong to a G-protein-coupled receptor superfamily and share a relatively higher homology with receptors for chemoattractants than those for other eicosanoids (21). Human BLT1 is expressed predominantly in leukocytes, whereas human BLT2 is expressed relatively ubiquitously (18, 20). In addition, two LTB4 receptors show different binding profiles to various eicosanoids and BLT antagonists. 12(S)-hydroxyeicosatetraenoic acid and 15(S)-hydroxyeicosatetraenoic acid bind to and activate BLT2 but not BLT1 (22). These eicosanoids also induce calcium mobilization and chemotaxis through BLT2 but not through BLT1 (22). In contrast to detailed studies on BLT1, information on BLT2 is limited. Thus, we cloned and characterized mouse BLT2 (mBLT2) to reveal the roles of BLT2 in vivo. In addition, we have developed a novel synthetic BLT2 agonist that selectively activates BLT2 in collaboration with Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Using this compound and a BLT1-specific antagonist, we demonstrated BLT2-dependent phosphorylation of extracellular signal-regulated kinase (ERK) and cell migration of primary mouse keratinocytes.

EXPERIMENTAL PROCEDURES

Materials—All of the eicosanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI). Compound A and its methyl ester derivative are gifts from Ono Pharmaceutical Co. Ltd., and CP105696 was from Pfizer Inc (Groton, CT). [3H]LTB4 was purchased from PerkinElmer Life Sciences.

Isolation of Genomic Clones Containing Mouse BLT1 and BLT2—Mouse genomic libraries were screened by plaque hybridization. 106
independent clones from mouse genomic library (129SV Mouse genomic library; Stratagene, La Jolla, CA) were lifted by Hybond N+ nylon membranes (Amersham Biosciences) and screened with a 1\(^{[32P]}\)dCTP-labeled probe, and positive clones were subjected to DNA sequencing (sequence data available from EMBL/GenBank\(^{TM}\)/DDBJ under accession number AA028322). Hybridization was carried out in a hybridization buffer containing 6\% sodium-sodium citrate solution (SSC), 10× Dehnhart’s solution, 0.5% SDS, and 100 μg/ml single-stranded salmon sperm DNA at 65 °C overnight. The membranes were washed in 2× SSC, 0.1× SSC followed by washing in 0.5× SSC, 0.1% SDS at 25 °C. Tertiary screening gave two positive clones, which were analyzed by Southern blotting. The DNA sequence was determined using an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). The DNA sequence was determined in both orientations. A positive clone contained 5.6 kb of mouse BLT1 upstream (1 bp from the first ATG), 5′-CTGCTGACGAAA-CACACTACATCTCT-3′, downstream (319), 5′-CAGTGGCATACTAGCTT-TATCAGC-3′; mBLT2 upstream (820), 5′-ACAGCTGTCGCCTCATCTCG-3′, downstream (1013), 5′-GGCCCAATTTCTTCAAGTT-3′; GAPDH upstream (1013), 5′-GTGGACCTCATGGCCTACAT-3′, downstream (1226), 5′-GGTGGCAGCGAAGTCTTTAG-3′. These primer pairs resulted in PCR products of 319 bp for mBLT1, 193 bp for mBLT2, and 1013 bp for GAPDH. The standards and samples were simultaneously amplified using the master mixture. The reactions were incubated at 95 °C for 10 min to activate the polymerase followed by 50 cycles of amplification. Each cycle of PCR consisted of 3 s of denaturation at 95 °C, 3 s of primer annealing at 65 °C, and 12 s of extension at 72 °C. The temperature ramp was set at 20 °C/s. At the end of each extension step, the fluorescent intensity was measured for quantification of cDNA. An exponential phase of the PCR products was obtained by a linear increase of the temperature to 95 °C. The levels of mRNA were normalized to the level of GAPDH mRNA.

Expression of Mouse BLT2 in CHO cells—The ORF of mBLT2 was amplified from isolated genomic clones by PCR using a sense primer (5′-GGGATCCGGATCCTGCTGGTGGTGGG-3′) covering a BamHI site and an antisense primer (5′-CCGAGGAGTTCCCTCGTGTTG-3′) covering a stop codon, followed by EcoRI site. The PCR products were digested with restriction enzymes and cloned into the BamHI/EcoRI site of pCOS1 (Invegnen) and designated pcDNA3-mBLT2. Accurate amplification of the insert was verified by DNA sequencing. CHO (Chinese hamster ovary) cells were maintained in Ham’s F-12 medium supplemented with 10% fetal calf serum (Sigma), 100 μg/ml streptomycin, and 100 IU/ml penicillin. For stable expression, CHO cells on 6-cm plates were transfected with 5 μg of plasmid DNA using Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol with 1 ml of PBS (Wako, Osaka, Japan) for 20 h. Resistant clones were isolated by limiting dilution, and expression of mBLT2 in these cells was confirmed by Northern blot analysis as described above. Several clones were examined for LTB4 binding and LTB4-dependent calcium mobilization, and they showed basically similar results.

\[^{[32P]}\]HTB4 Binding Assay—Cells were harvested and sonicated in a buffer containing 20 mM Tris-HCl, pH 7.4, 0.25 mM sucrose, 10 mM MgCl$_2$, 2 mM EDTA-Na$_2$, 2 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin A. After centrifugation at 12,000 × g for 10 min at 4 °C, the remaining supernatants were further centrifuged at 105,000 × g for 60 min. The pellets were used for binding assays as membrane fractions. The binding mixture (100 μl) contained membrane fractions (20 μg of protein) and 0.5 nM \[^{[32P]}\]HLTB4 (17,760 dpm for membrane fractions of CHO-mBLT1) or 5 nM \[^{[32P]}\]HLTB4 (177,600 dpm for membrane fractions of CHO-mBLT2) in binding buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, 10 mM NaCl, and 0.1% BSA). For determination of the nonspecific binding, unlabeled LTB4 was added to the binding mixture to a final concentration of 1 μM. The mixtures were incubated at room temperature for 60 min by gentle agitation followed by rapid filtration through GF/C filters (PerkinElmer Life Sciences) and extensive washing with 3 ml of binding buffer. The radioactivity of the filters was determined with a Top Count scintillation counter (PerkinElmer Life Sciences).

**Measurements of Calcium and cAMP Concentrations—**Calcium and cAMP concentrations were determined according to the previous method (20) using CHO (mock-transfected), CHO-mBLT1, and CHO-mBLT2 cells using a CAP-100 system (Jasco, Tokyo, Japan) and Bio-Track camp enzyme immunoassay system (Amersham Biosciences). To examine the inhibitory effect of CP105966 on LTB4-induced Ca$^{2+}$ mobilization, CP105966 was applied 1 min before 100 nM LTB4 application.

**ERK Phosphorylation—**CHO cells were seeded on 12-well plates at 2 × 10$^5$ cells/well. After 2 days of culture, the cells were serum-starved for 12 h in Ham’s F-12 medium containing 0.1% bovine serum albumin (BSA) (Fraction V). The cells were preincubated with Ham F-12 containing 0.1% BSA at 37 °C for 10 min and then exposed with various concentrations of ligands for 5 min. Pilot experiments showed that 5 μM of stimulation gave maximum phosphorylation of ERK by LTB4 and Compound A. Primary mouse keratinocytes were isolated as described (24) and were seeded on 12-well plates at 4 × 10$^5$ cells/well. After 1 day of culture, the cells were incubated for 12 h in Defined keratinocyte SFM (serum-free medium) (Invitrogen). The cells were preincubated with Defined keratinocyte SFM (serum-free medium) containing 0.1% BSA at 37 °C for 30 min and stimulated as described above. After 10 min, 100 nM LTB4 was applied to the cells. The medium was removed, and the cells were lysed in 250 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 50 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na$_2$VO$_3$, 2 mM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) frozen at −80 °C, thawed, and homogenized by passing through a 27-gauge needle 10 times. The cell lysate was denatured at 100 °C for 10 min and separated in 12% SDS-PAGE gels. After transfer to nitrocellulose membranes (Hybond ECL) using semi-dry transfer cell (Bio-Rad) at 350 mA for 20 min. The membranes were blocked overnight at 4 °C with the blocking solution (Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) skim milk). They were then incubated for 2 h with a dilution of (1:1000) anti-phospho-p44/p42 MAPK antibody (code 9101, Cell signaling, Beverly, MA) in Tris-buffered saline containing 200 μg/ml BSA and 0.1% Tween 20 and 5% (w/v) skim milk. For the total ERK, they were incubated for 2 h with a dilution of (1:1000) anti-p44/p42 MAPK antibody (code 9102, Cell Signaling) in dilution solution (Tris-buffered saline containing 0.1% (v/v) Tween 20 and 0.1% (w/v) BSA). Horseradish peroxidase-conjugated secondary antibody (code 7074, Cell Signaling) was used in both cases at a dilution of 2000 for 1 h followed by development using ECL (Amersham Biosciences).

**In Situ Hybridization—**In situ hybridization for mBLT2 was carried out using paraffin sections of skin samples from C57Bl/6j mice fixed in 10% formalin/phosphate-buffered saline without calcium chloride and magnesium (PBS−) as described previously (25) with slight modifications. Briefly, paraffin-embedded tissues were cut into 4-μm-thick sections, mounted onto silane-coated slides, deparaffinized, and treated with proteinase K (5 μg/ml in PBS−) for 20 min at room temperature and with 2 μg/ml glycine in PBS− for 15 min at room temperature. The sections were acidified with acetic anhydride (1 ml in 400 ml of 0.1% triethanolamine, pH 8.0) for 15 min at room temperature. After washing with PBS−, the samples were soaked in 2× SSC containing 50% formamide and subjected to hybridization. A fragment of mBLT2 coding region (1165 bp) was ampliﬁed by PCR using primers with a recognition sequence for HindIII and a downstream primer with a recognition sequence for EcoRI and then directionally subcloned into pSP178 (Roche Applied Science). The plasmid was linearized using HindIII to prepare the antisense probe and EcoRI for the sense probe. The probes were labeled with digoxigenin-11-UTP using a digoxigenin RNA labeling kit (Roche Applied Science). Hybridization was performed in a humidified chamber for 16 h at 42 °C. The slides were washed in 2× saline-sodium citrate (SSC), 0.1% SDS at 25 °C.

**Functional Leukotriene B4 Receptor 2 in Primary Keratinocytes**

24187
Functional Leukotriene B4 Receptor 2 in Primary Keratinocytes

FIG. 1. Calcium mobilization in CHO-mBLT1 and -mBLT2. A, increase in intracellular calcium after exposure to various concentrations of LTB4 was measured in CHO-mBLT1 (●) and CHO-mBLT2 (■). Data are represented as percentages of maximum responses (mean ± S.D., n = 3). EC50 values of LTB4 were 20 and 170 nM for mBLT1 and mBLT2, respectively. CHO cells transfected with an empty vector did not respond to 10 μM LTB4 (▲). B, inhibitory effect of a BLT antagonist, CP105696, on 100 nM LTB4-induced calcium mobilization in CHO-mBLT1 (●) and CHO-mBLT2 (■). CP105696 antagonized BLT1 in a dose-dependent manner but did not antagonize BLT2. Similar results were obtained in an additional experiment. Maximum calcium responses in CHO-mBLT1 and CHO-mBLT2 were 410 and 640 nM, respectively.

FIG. 2. cAMP accumulation in forskolin-treated CHO-mBLT1 and -mBLT2. A, inhibitory effects of LTB4 on 25 μM forskolin-induced cAMP accumulation in CHO-mBLT1 (●), CHO-mBLT2 (■), and mock transfecants (▲) (mean ± S.D., n = 3). IC50 values of LTB4 in inhibiting adenylyl cyclase activity in CHO-mBLT1 and CHO-mBLT2 were 1 and 80 nM, respectively. Forskolin-induced cAMP levels in the absence of LTB4 were 49.6 ± 1.6 pmol/106 CHO-mBLT1 and 45.9 ± 0.9 pmol/106 CHO-mBLT2 (mean ± S.D., n = 3). The results are representative of three independent experiments. B, inhibitory effect of PTX pretreatment (100 ng/ml, 12 h) on adenylyl cyclase inhibition by LTB4 in CHO-mBLT2. 25 μM forskolin-induced cAMP levels in the absence of LTB4 were 72.1 ± 1.6 pmol/106 untreated cells (■) and 24.7 ± 0.3 pmol/106 PTX-treated cells (●) (mean ± S.D., n = 3). Similar results were obtained in an additional experiment.

RESULTS

Pharmacological Properties of mBLT2—To clone and analyze mouse BLT2 (mBLT2), we screened mouse genomic library and isolated several clones containing both mBLT2 and mBLT1 genes. Southern blot and DNA sequencing analyses revealed that the gene organization of mBLT1 and mBLT2 was quite similar (data not shown) to that of human (26). mBLT2 was similar to human BLT2 with identities of 92.2 and 86.0% on the amino acid and nucleotide levels, respectively. Homology between human and mouse BLT2 is much higher than BLT1 (78.6% amino acid identity), suggesting that BLT2 has been conserved during evolution. The membrane fraction of human...
Compound A is a selective agonist for BLT2. A, the structure of Compound A. B, inhibition of [3H]LTB4 binding to BLT1 by LTB4 (●) and Compound A (▲). 0.5 nM [3H]LTB4 binding to membrane preparation (20 μg of protein) of CHO-mBLT1 was not inhibited by Compound A. The carboxyl methyl ester derivative of Compound A (□) did not inhibit [3H]LTB4 binding to mBLT1. C, inhibition of [3H]LTB4 binding to BLT2 by LTB4 (●) and Compound A (▲). 5 nM [3H]LTB4 binding to membrane preparation (20 μg of protein) of CHO-mBLT2 was inhibited by LTB4 and Compound A in dose-dependent manners. The carboxyl methyl ester derivative of Compound A (□) did not inhibit [3H]LTB4 binding to mBLT2. D, increase in intracellular calcium after exposure to various concentrations of Compound A was measured in CHO-mBLT1 (○) and CHO-mBLT2 (▲). Although Compound A increased intracellular calcium concentrations in CHO-mBLT2 dose-dependently, there was no effect in CHO-mBLT1 or mock-transfected cells (data not shown). The carboxyl methyl ester derivative of Compound A (10 μM) did not increase intracellular calcium either in CHO-mBLT1 (X) or in CHO-mBLT2 (□). The EC50 value of Compound A (▲) is 20 nM, which is lower than that of LTB4 (●) (170 nM) in CHO-mBLT2. Data are represented as percentages of maximum responses (mean ± S.D., n = 3). E and F, ERK phosphorylation in CHO-mBLT2 and mBLT1. Both LTB4 and Compound A induced ERK phosphorylation (pERK) in CHO-mBLT2 (E). LTB4 induced ERK phosphorylation in CHO-mBLT1, but Compound A did not (F). All experiments were performed at least twice with similar results.

Embryonic kidney (HEK)-293 cells transfected with mBLT2 showed specific [3H]LTB4 binding (data not shown).

To examine whether the binding of LTB4 to mBLT2 transduces intracellular signaling, we established several lines of CHO cells stably expressing mBLT2 (CHO-mBLT2) and compared the dose dependence of LTB4-induced signaling in CHO-mBLT2 to that in CHO-mBLT1. LTB4 increased intracellular calcium concentrations in dose-dependent manners for both CHO-mBLT1 and CHO-mBLT2 (Fig. 1A). EC50 values of LTB4 were 20 nM for mBLT1 and 170 nM for mBLT2 (Fig. 1A). 10 μM LTB4 did not cause any calcium increase in CHO cells transfected with an empty vector. We examined the inhibitory effect of a BLT antagonist, CP105696 (11), on LTB4-induced calcium mobilization in CHO-mBLT1 and CHO-mBLT2. CP105696 inhibited LTB4-induced calcium mobilization in CHO-mBLT1 in a dose-dependent manner but did not in CHO-mBLT2 (Fig. 1B), showing that this compound is a BLT1-specific antagonist.

We next examined the inhibitory effects of LTB4 on adenyl cyclase activity through mBLT1 and mBLT2. LTB4 dose-dependently inhibited 25 μM forskolin-activated adenyl cyclase in both CHO-mBLT1 and CHO-mBLT2 (Fig. 2A). The IC50 value of LTB4 in inhibiting adenyl cyclase activity in CHO-mBLT2 (80 nM; Fig. 2A) was higher than that of CHO-mBLT1 (1 nM; Fig. 2A). Pretreatment of cells with 100 ng/ml pertussis toxin (PTX) for 12 h abolished LTB4-dependent adenyl cyclase inhibition in CHO-mBLT2 cells (Fig. 2B), showing that mBLT2 couples with the G/o class of G-protein in inhibiting adenyl cyclase. All these data show that mouse BLT2 is a functional and low affinity receptor for LTB4.

Selective BLT2 Agonist, Compound A—Compound A, 4’-(pentanoyl (phenyl) amino) methyl)-1, 1’-biphenyl-2-carboxylic acid (Fig. 3A), was identified as a BLT2 agonist in the chemical library of Ono Pharmaceutical Co. Ltd. To test the specificity of this compound on BLT1 and BLT2, we examined the competitive effects of Compound A on [3H]LTB4 binding to membrane fractions of CHO-mBLT1 or CHO-mBLT2. Fig. 3B shows the competition of 0.5 nM [3H]LTB4 binding to mBLT1 by various concentrations of unlabeled LTB4 and Compound A. LTB4 binding to BLT1 was inhibited by unlabeled LTB4 by a dose-dependent manner (IC50 value, 50 nM) but not by compound A (Fig. 3B). Fig. 3C shows the competition of 5 nM [3H]LTB4 binding to mBLT2 by various concentrations of unlabeled LTB4 and Compound A. In contrast to BLT1, LTB4 binding to BLT2 was inhibited by both unlabeled LTB4 and Compound A. IC50 values of LTB4 and Compound A were around 50 and 20 nM, respectively. 1 μM Compound A inhibited 77% of [3H]LTB4 binding to BLT2 (Fig. 3C). The carboxyl methyl ester derivative of Compound A did not inhibit [3H]LTB4 binding to BLT1 or BLT2 (Fig. 3, B and C). We also examined the agonistic activity of this compound using CHO-mBLT1 and CHO-mBLT2. Compound A increased the intracellular calcium concentration in CHO-mBLT2 in a dose-dependent manner but not in CHO-mBLT1 or mock-transfected cells (Fig. 3D). The carboxyl methyl ester derivative of Compound A did not increase intracellular calcium after exposure to various concentrations of Compound A was measured in CHO-mBLT1 (○) and CHO-mBLT2 (▲). Although Compound A increased intracellular calcium concentrations in CHO-mBLT2 dose-dependently, there was no effect in CHO-mBLT1 or mock-transfected cells (data not shown). The carboxyl methyl ester derivative of Compound A (10 μM) did not increase intracellular calcium either in CHO-mBLT1 (X) or in CHO-mBLT2 (□). The IC50 value of Compound A (▲) is 20 nM, which is lower than that of LTB4 (●) (170 nM) in CHO-mBLT2. Data are represented as percentages of maximum responses (mean ± S.D., n = 3). E and F, ERK phosphorylation in CHO-mBLT2 and mBLT1. Both LTB4 and Compound A induced ERK phosphorylation (pERK) in CHO-mBLT2 (E). LTB4 induced ERK phosphorylation in CHO-mBLT1, but Compound A did not (F). All experiments were performed at least twice with similar results.

Embryonic kidney (HEK)-293 cells transfected with mBLT2 showed specific [3H]LTB4 binding (data not shown).

To examine whether the binding of LTB4 to mBLT2 transduces intracellular signaling, we established several lines of CHO cells stably expressing mBLT2 (CHO-mBLT2) and compared the dose dependence of LTB4-induced signaling in CHO-mBLT2 to that in CHO-mBLT1. LTB4 increased intracellular calcium concentrations in dose-dependent manners for both CHO-mBLT1 and CHO-mBLT2 (Fig. 1A). EC50 values of LTB4 were 20 nM for mBLT1 and 170 nM for mBLT2 (Fig. 1A). 10 μM LTB4 did not cause any calcium increase in CHO cells transfected with an empty vector. We examined the inhibitory effect of a BLT antagonist, CP105696 (11), on LTB4-induced calcium mobilization in CHO-mBLT1 and CHO-mBLT2. CP105696 inhibited LTB4-induced calcium mobilization in CHO-mBLT1 in a dose-dependent manner but did not in CHO-mBLT2 (Fig. 1B), showing that this compound is a BLT1-specific antagonist.

We next examined the inhibitory effects of LTB4 on adenyl cyclase activity through mBLT1 and mBLT2. LTB4 dose-dependently inhibited 25 μM forskolin-activated adenyl cyclase in both CHO-mBLT1 and CHO-mBLT2 (Fig. 2A). The IC50 value of LTB4 in inhibiting adenyl cyclase activity in CHO-mBLT2 (80 nM; Fig. 2A) was higher than that of CHO-mBLT1 (1 nM; Fig. 2A). Pretreatment of cells with 100 ng/ml pertussis toxin (PTX) for 12 h abolished LTB4-dependent adenyl cyclase inhibition in CHO-mBLT2 cells (Fig. 2B), showing that mBLT2 couples with the G/o class of G-protein in inhibiting adenyl cyclase. All these data show that mouse BLT2 is a functional and low affinity receptor for LTB4.

Selective BLT2 Agonist, Compound A—Compound A, 4’-(pentanoyl (phenyl) amino) methyl)-1, 1’-biphenyl-2-carboxylic acid (Fig. 3A), was identified as a BLT2 agonist in the chemical library of Ono Pharmaceutical Co. Ltd. To test the specificity of this compound on BLT1 and BLT2, we examined the competitive effects of Compound A on [3H]LTB4 binding to membrane fractions of CHO-mBLT1 or CHO-mBLT2. Fig. 3B shows the competition of 0.5 nM [3H]LTB4 binding to mBLT1 by various concentrations of unlabeled LTB4 and Compound A. LTB4 binding to BLT1 was inhibited by unlabeled LTB4 in a dose-dependent manner (IC50 value, 50 nM) but not by compound A (Fig. 3B). Fig. 3C shows the competition of 5 nM [3H]LTB4 binding to mBLT2 by various concentrations of unlabeled LTB4 and Compound A. In contrast to BLT1, LTB4 binding to BLT2 was inhibited by both unlabeled LTB4 and Compound A. IC50 values of LTB4 and Compound A were around 50 and 20 nM, respectively. 1 μM Compound A inhibited 77% of [3H]LTB4 binding to BLT2 (Fig. 3C). The carboxyl methyl ester derivative of Compound A did not inhibit [3H]LTB4 binding to BLT1 or BLT2 (Fig. 3, B and C). We also examined the agonistic activity of this compound using CHO-mBLT1 and CHO-mBLT2. Compound A increased the intracellular calcium concentration in CHO-mBLT2 in a dose-dependent manner but not in CHO-mBLT1 or mock-transfected cells (Fig. 3D). The carboxyl methyl ester derivative of Compound A did not increase intra-
cellular calcium concentration in either CHO-mBLT1 or CHO-mBLT2 (Fig. 3D). The maximum response evoked by Compound A was similar to that of LT4, suggesting that Compound A is a full agonist for mBLT2. The EC_{50} value of Compound A for mBLT2 is 20 nM, which is lower than that of LT4 (170 nM), as determined by calcium mobilization assay. The potency and selectivity of Compound A on human BLT2 were similar for that on mouse BLT2, and Compound A did not induce calcium mobilization through human BLT1 (data not shown). Thus, we concluded that Compound A is a selective and full BLT2 agonist and activates BLT2 at lower concentrations than LT4. Next, we examined LT4- and Compound A-dependent phosphorylation of ERK. LT4 dose-dependently induced ERK phosphorylation both in CHO-mBLT1 and in CHO-mBLT2 (Fig. 3E, F, left). Minimum concentrations of LT4 required for ERK phosphorylation were 1 nM in CHO-mBLT1 (Fig. 3F, left) and 10 nM in CHO-mBLT2 (Fig. 3E, left). Compound A induced ERK phosphorylation only in CHO-mBLT2 but not in CHO-mBLT1 (Fig. 3, E and F, right). The minimum concentration of Compound A required for ERK phosphorylation in CHO-mBLT2 was 1 nM.

**Agonistic Activity of 12-epi LT4 against BLTs**—We next examined whether dihydroxyeicosatetraenoic acids could activate mBLT1 and mBLT2. [3H]LT4 binding to BLT1 was inhibited by 12-epi LT4 but not by 6-trans-12-epi LT4 (Fig. 4A). The IC_{50} value of 12-epi LT4 (70 nM) was higher than that of LT4 (3 nM). [3H]LT4 binding to BLT2 was inhibited by 12-epi LT4 but not by 6-trans-12-epi LT4 (Fig. 4B). The IC_{50} value of 12-epi LT4 (70 nM) was similar to that of LT4 (50 nM). 12-epi LT4 increased intracellular calcium concentrations in CHO-mBLT1 and CHO-mBLT2, whereas 6-trans-12-epi LT4 did not (Fig. 4, C and D). The EC_{50} values of 12-epi LT4 were 170 and 300 nM for mBLT1 and mBLT2, respectively. The maximum calcium mobilization induced by 12-epi LT4 in CHO-mBLT1 was about 50% of that by LT4 and about 80% for CHO-mBLT2 (Fig. 4, C and D). In addition, 12-epi LT4 induced ERK phosphorylation both in CHO-mBLT1 and in CHO-mBLT2 (Fig. 4, E and F, left), whereas 6-trans-12-epi LT4 did not (Fig. 4, E and F, right).

**Tissue Distribution**—Northern blot analysis using mouse tissues showed that mBLT2 mRNA was expressed in small intestine and skin (Fig. 5A). The sizes of major transcripts were 1.5 and 6.7 kb. Quantitative real time RT-PCR showed the highest expression in colon and spleen (Fig. 5B). The expression levels of mBLT2 in macrophages and neutrophils were under the detection limit. Tissue distribution of mBLT2 is quite different from that of human BLT2, which is expressed rather ubiquitously, having the most abundant expression in spleen followed by liver, ovary, and leukocytes (26). The expression of mBLT2 is different from that of mBLT1, which was reported to be expressed predominantly in macrophages, eosinophils, and neutrophils (27). To determine which cells express mBLT2, in situ hybridization was performed with mouse skin. In skin, BLT2 mRNA was detected in follicular and interfollicular keratinocytes (Fig. 6, A and C). No signal was obtained using the...
sense control probe (Fig. 6, B and D). We could not identify BLT2-expressing cells in mouse small intestine because of the high background in this tissue (data not shown).

**LTB4 Induced ERK Phosphorylation and Cell Migration through BLT2 in Primary Mouse Keratinocytes—**In situ hybridization in mouse skin showed that BLT2 was expressed in epidermal keratinocytes (Fig. 6). Accordingly, we isolated primary keratinocytes from mouse skin and examined them for ERK phosphorylation and cell migration induced by LTB4 and Compound A. Staining with anti-mouse keratin 5 antibody showed that the purity of isolated keratinocytes was more than 98% (Fig. 7A). We also confirmed the expression of BLT1 and BLT2 in isolated keratinocytes by RT-PCR (Fig. 7B). LTB4, 12-epi LTB4, and Compound A dose-dependently induced ERK phosphorylation in primary mouse keratinocytes (Fig. 7C). 100 nM Compound A caused full phosphorylation of ERK. ERK phosphorylation in keratinocytes induced by 100 nM LTB4 was not inhibited by 10 μM CP105696 (BLT1 specific antagonist, Fig. 1B) (Fig. 7D). Both LTB4 and ONO L047 induced keratinocyte migration in dose-dependent manners (Fig. 7, E and F), and the dose of LTB4 required for keratinocyte migration was as high as 1 μM. These data suggest the presence of functional BLT2 in mouse primary keratinocytes.

**DISCUSSION**

LTB4 has long been known as a potent lipid chemoattractant for phagocytes and is believed to be involved in various inflammatory diseases (9–13). Many BLT antagonists had been developed without knowing the structural information of LTB4 receptors, and no BLT antagonists are currently available for clinical usage. Since the identification of BLT1 and BLT2, high and low affinity LTB4 receptors, these classical BLT antagonists have been under re-evaluation for their efficacy on two types LTB4 receptors. Most of these antagonists preferentially antagonize BLT1 (Fig. 2B) (22), and this is reasonable because they were developed using granulocytes, which predominantly express BLT1 as target cells. Several lines of genetically engineered mice with BLT1 have been established and analyzed. BLT1 transgenic mice with the β2 integrin promoter developed severe lung injury after hind limb-ischemic reperfusion (28). BLT1-deficient mice exhibited milder peritonitis with reduced infiltration of inflammatory cells (29), reduced formation of atherosclerotic plaques on ApoE(−/−) background (30), and attenuated recruitments of CD4+ (15) and CD8+ (16) T cells into inflamed lesion. In contrast to extensive studies on BLT1, only limited information is available on BLT2. Detailed analysis of mouse BLT2 is important to reveal the in vivo roles of BLT2 in various disease models using genetically engineered mice.

We first isolated mouse genomic clones containing genes for BLT1 and BLT2 because human BLT1 and BLT2 form a gene cluster on chromosome 14 (20, 26). We successfully isolated several clones containing both mouse BLT1 and BLT2 genes. The order and orientation of mouse genes for BLT1 and BLT2 and their neighboring genes for CIDE-B (cell-death induced DFF45-like effector) (31) and adenyllyl cyclase IV are quite similar to those for human (data not shown). DNA sequencing suggested that the ORF for mBLT2 is intronless, so we constructed expression vectors for mBLT2 using the isolated genomic clone.

We established CHO cells stably expressing mBLT1 or mBLT2 (CHO-mBLT1 and CHO-mBLT2) and examined intracellular signaling by LTB4. In the calcium mobilization assay, EC_{50} values of LTB4 in CHO-mBLT1 and CHO-mBLT2 cells were 20 and 170 nM, respectively (Fig. 1A). A classical BLT antagonist, CP105696, dose-dependently inhibited LTB4-in-
Although LTB4 stimulation caused significant intracellular signaling in some native cells (32–34), it was difficult to determine whether LTB4 signaling is mediated by BLT1 or BLT2 or both. This was due to the lack of selective agonists or antagonists that distinguish BLT1 and BLT2. During screening of BLT2 antagonists in collaboration with Ono Pharmaceutical Co. Ltd., we identified a synthetic compound, Compound A, which specifically activates BLT2 but not BLT1. Binding assays using fractionated cell membranes showed that Compound A inhibited [3H]LTB4 binding to BLT2 but not BLT1 (Fig. 3, B and C). Compound A inhibited 77% of [3H]LTB4 binding to mBLT2 (Fig. 3C). An IC50 value of Compound A on [3H]LTB4 binding to BLT2 was almost similar to that of unlabeled LTB4. Compound A dose-dependently induced calcium mobilization and ERK phosphorylation in CHO-mBLT2, whereas it had no effects in CHO-mBLT1 or mock transfectants (Fig. 3D). EC50 values of Compound A in calcium mobilization and ERK phosphorylation in CHO-mBLT2 were lower than those of LTB4 by 10-fold. The maximum calcium responses obtained by Compound A and LTB4 were comparable (Fig. 3D). These data clearly illustrate that Compound A is a selective and full agonist for mBLT2. We have confirmed that Compound A is also a potent and selective agonist for human BLT2 (data not shown). Free carboxylic acid of Compound A is necessary for binding to BLT2 because the carboxyl methyl ester derivative of Compound A did not bind to or activate mBLT2 at all (Fig. 3, C and D). The selectivity of this compound on BLT2 promotes us to explore in vivo roles of BLT2 in future studies. The mechanism of more efficient signaling from BLT2 activated by Compound A rather than by LTB4 (despite the same binding potency remains) remains to be elucidated. 6-trans-12-epi-LTB4, a non-enzymatic hydrolysis product of LTA4, did not activate mBLT1 or BLT2. However, its isomer 12-epi-LTB4 activated both mBLT1 and mBLT2, leading to calcium mobilization and ERK phosphorylation (Fig. 4, A–F). Thus, the 6-cis configuration of LTB4 appears important in recognition by mBLT1 and mBLT2.

We next precisely determined BLT2 expression in various mouse tissues and cells. Northern blot and quantitative RT-PCR analyses showed that mBLT2 mRNA is expressed highly in small intestine and skin of C57Bl/6J mice (Fig. 5, A and B). Human BLT2 is expressed relatively ubiquitously with the highest expression in spleen followed by liver, ovary, and peripheral leukocytes (20). We have no clear explanation on the different expression profiles of BLT2 in human and mouse (Fig. 5A). Detailed analyses of BLT2 promoters in human and mouse are required to determine the molecular mechanism regulating tissue-specific expression of BLT2.

By in situ hybridization, we found that mBLT2 is expressed in follicular and interfollicular keratinocytes (Fig. 6). Mouse keratinocytes express both BLT1 and BLT2, as revealed by RT-PCR (Fig. 7B), and LTB4, 12-epi-LTB4, and Compound A dose-dependently induced ERK phosphorylation in these cells (Fig. 7C), suggesting involvement of BLT2 in ERK phosphorylation. To confirm BLT2-dependent ERK phosphorylation, we tried to antagonize BLT1 in keratinocytes. A BLT1-specific antagonist, CP105696 (Fig. 1B), at 10 μM did not inhibit 100 nM LTB4-induced ERK phosphorylation in keratinocytes (Fig. 7D). All these data suggest that activation of BLT2 is sufficient for LTB4-ERK phosphorylation in primary keratinocytes, although we are unable to exclude the possibility of BLT2-independent activity of Compound A. Furthermore, LTB4 and Compound A induced cell migration in primary mouse keratinocytes (Fig. 7, E and F). The dose of LTB4 required for kerato-
tinocyte migration (Fig. 7E) was similar to that for calcium mobilization in CHO-mBLT2 cells (Fig. 1A). Compound A induced keratinocyte migration at lower doses than LTB4 (Fig. 7F), as is the case for calcium mobilization in CHO-mBLT2 cells. Thus, we conclude that mouse keratinocytes express functional BLT2. Keratinocytes are the major population of epidermis and the first barrier of protection against invasion of foreign substances into the body, and they are known to produce proinflammatory and immunomodulatory cytokines and chemokines against various stimuli (35). Keratinocytes were reported to express both 5-lipoxgenase (36) and LTA4 hydroxase (37), both required for LTB4 biosynthesis. Skin is also a site for production of various types of monohydroxy fatty acids (38–40), which are known as ligands for BLT2 (22). Several studies have suggested roles of LTB4 in psoriasis, an inflammatory skin disease, and the itch-associated response in mice (41, 42). BLT2 may have some roles in proliferation and maturation of keratinocytes and initiation of inflammation and immune responses (43) in skin by activating keratinocytes.

In conclusion, we have cloned and characterized mouse BLT2 as a functional low affinity receptor for LTB4. We detected the expression of mBLT2 and intracellular signaling through intrinsic BLT2 in primary mouse keratinocytes by the use of a BLT2 selective agonist, Compound A, and a BLT1-specific antagonist. These findings will be useful in interpreting and understanding the pathophysiological roles of the LTB4-BLT2 axis in vivo.

Acknowledgments—We are grateful to Dr. Y. Ide (Keio University) for confocal microscopic analyses, Drs. S. Ishii and S. Yano (The University of Tokyo), and all members in our laboratory for discussion and suggestions. Also, we thank Ono Pharmaceutical Co. Ltd. and Pfizer Inc. for materials.

REFERENCES

1. Samuelsson, B., Dahlen, S. E., Lindgren, J. A., Rouzer, C. A., and Serhan, C. N. (1987) Science 237, 1171–1176
2. Marshall, J. K., Krump, E., Lindsay, T., Downey, G., Ford, D. A., Zhu, P., Walker, P., and Rubin, B. (2000) J. Immunol. 164, 2084–2091
3. Shimizu, T., Izumi, T., Seyama, Y., Takeda, K., Radmark, O., and Samuelsson, B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4175–4179
4. Matsumoto, T., Funk, C. D., Radmark, O., Hoog, J. O., Jornvall, H., and Samuelsson, B. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 26–30
5. Minami, M., Ohno, S., Kawasaki, H., Shimizu, T., and Izumi, T. (2002) J. Biol. Chem. 277, 18763–18768
6. Yamasaki, K., Torii, N., Hanakawa, Y., Shirakata, Y., Sayama, K., Takeyanagi, A., Ohtsubo, M., Gamou, S., Shimizu, N., Fujii, M., Miyazono, K., and Hashimoto, K. (2003) J. Invest. Dermatol. 120, 1030–1037
7. Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ito, H., Fujimoto, M., Grotendorst, G. R., and Takehara, K. (1996) J. Invest. Dermatol. 106, 729–733
8. Kato, K., Yokomizo, T., Izumi, T., and Shimizu, T. (2000) J. Exp. Med. 192, 413–420
9. Huang, W. W., Garcia-Zepeda, E. A., Sauty, A., Oettgen, H. C., Rothenberg, M. E., and Luster, A. D. (1998) J. Exp. Med. 186, 1063–1074
10.栈田, G., 栃原, K., Clish, C. B., O'Brien, J. A., Freeman, M. W., and Subbarao, K. (1998) J. Immunol. 160, 2939–316
11. Maruyama, M., and Kuraishi, Y. (2001) J. Exp. Med. 192, 433–438
12. Subbarao, K., Jala, V. R., Mathis, S., Suttles, J., Zacharias, W., Ahamed, J., Ali, H., Tseng, M. T., and Haribabu, B. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 369–375
13. Inohara, N., Koseki, T., Chen, S., Wu, X., and Nunez, G. (1998) EMBO J. 17, 2526–2533
14.栈田, K., Hoo, M. H., You, H. J., Cho, S. H., Mun, Y. C., Seong, C. M., and Kim, J. H. (2000) J. Immunol. 164, 6723–6729
15.栈田, H., You, H. J., Cho, S. H., Eom, Y. W., Chun, J. S., You, Y. J., and Kim, J. H. (2002) J. Biol. Chem. 277, 8572–8578
16. Lindsay, M. A., Haddad, E. B., Russell, J., Teixeira, M. M., Hellewell, P. G., Barnes, P. J., and Giembycz, M. A. (1998) J. Leukocyte Biol. 64, 555–562
17. Stackstra, K., Torii, N., Hanakawa, Y., Shirakata, Y., Sayama, K., Takeyanagi, A., Ohtsubo, M., Gamou, S., Shimizu, N., Fujii, M., Miyazono, K., and Hashimoto, K. (2003) J. Invest. Dermatol. 120, 1030–1037
18. Kato, K., Yokomizo, T., Izumi, T., and Shimizu, T. (2000) J. Exp. Med. 192, 413–420
19. Huang, W. W., Garcia-Zepeda, E. A., Sauty, A., Oettgen, H. C., Rothenberg, M. E., and Luster, A. D. (1998) J. Exp. Med. 186, 1063–1074
20. 案田, G., 栃原, K., Clish, C. B., O’Brien, J. A., Freeman, M. W., and Subbarao, K. (1998) J. Immunol. 160, 2939–316
21. Maruyama, M., and Kuraishi, Y. (2001) J. Exp. Med. 192, 433–438
22. Subbarao, K., Jala, V. R., Mathis, S., Suttles, J., Zacharias, W., Ahamed, J., Ali, H., Tseng, M. T., and Haribabu, B. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 369–375
23. Inohara, N., Koseki, T., Chen, S., Wu, X., and Nunez, G. (1998) EMBO J. 17, 2526–2533
24.栈田, H., You, M. H., You, H. J., Cho, S. H., Mun, Y. C., Seong, C. M., and Kim, J. H. (2000) J. Immunol. 164, 6723–6729
25.栈田, H., You, H. J., Cho, S. H., Eom, Y. W., Chun, J. S., You, Y. J., and Kim, J. H. (2002) J. Biol. Chem. 277, 8572–8578
26. Lindsay, M. A., Haddad, E. B., Russell, J., Teixeira, M. M., Hellewell, P. G., Barnes, P. J., and Giembycz, M. A. (1998) J. Leukocyte Biol. 64, 555–562
27. Block, C., Tumin, U., Vickers, P. J., Wittig, U., Lehmann, W. D., Stark, H. J., Fusenig, N. E., Rosenbach, T., Radmark, O., Samuelsson, B., and Habe nick, N. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6966–6970
28. Iversen, L., Svendsen, M., and Kragballe, K. (1996) Acta Dermatovenerol. 76, 424–428
29. Boeglin, W. E., Kim, R. B., and Brash, A. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6744–6749
30. Hassain, H., Figures, L. P., Shannon, V. R., Wilson, J. D., Funk, C. D., Pentland, A. P., and Holtzman, M. J. (1994) Am. J. Physiol. 266, C243–C253
31. Ando, H., Yagita, Y., Takahisa, H., and Kuraishi, Y. (2004) J. Invest. Dermatol. 123, 196–201
32. Ando, H., Katsube, N., Maruyama, M., and Kuraishi, Y. (2001) J. Invest. Dermatol. 117, 1621–1626
33. Mackenzie, R. H., Sewell, H. J., Dawson, A. A., Ratcliffe, M. A., Bennett, N. B., and King, D. J. (1999) Dis. Markers 8, 137–143
