A Second Leukotriene B_4 Receptor, BLT2: A New Therapeutic Target in Inflammation and Immunological Disorders

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Abstract

Leukotriene B_4 (LTB_4) is a potent chemoattractant and activator of both granulocytes and macrophages. The actions of LTB_4 appear to be mediated by a specific G protein–coupled receptor (GPCR) BLT1, originally termed BLT (Yokomizo, T., T. Izumi, K. Chang, Y. Takuwa, and T. Shimizu. 1997. *Nature*. 387:620–624). Here, we report the molecular cloning of a novel GPCR for LTB_4, designated BLT2, which binds LTB_4 with a Kd value of 23 nM compared with 1.1 nM for BLT1, but still efficiently transduces intracellular signaling. BLT2 is highly homologous to BLT1, with an amino acid identity of 45.2%, and its open reading frame is located in the promoter region of the BLT1 gene. BLT2 is expressed ubiquitously, in contrast to BLT1, which is expressed predominantly in leukocytes. Chinese hamster ovary cells expressing BLT2 exhibit LTB_4-induced chemotaxis, calcium mobilization, and pertussis toxin–insensitive inhibition of adenylyl cyclase. Several BLT1 antagonists, including U 75302, failed to inhibit LTB_4 binding to BLT2. Thus, BLT2 is a pharmacologically distinct receptor for LTB_4, and may mediate cellular functions in tissues other than leukocytes. BLT2 provides a novel target for antiinflammatory therapy and promises to expand our knowledge of LTB_4 function. The location of the gene suggests shared transcriptional regulation of these two receptors.

Key words: BLT • cloning • gene cluster • leukotriene • low-affinity receptor

Introduction

Leukotriene B_4 (LTB_4;[1] 5[*S*],12[*R*]-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid) is a metabolite of arachidonic acid and is one of the most potent activators of granulocytes and macrophages (1–3). LTB_4 binds to a specific G protein–coupled receptor (GPCR) named BLT and activates the Gi and G16 classes of G proteins (4) to inhibit adenylyl cyclase and activate phospholipase C. Exposure to LTB_4 induces adhesion of granulocytes to endothelial cells, degranulation of the lysosomal enzymes, generation of superoxide, and transmigration of granulocytes, all important in the host defense against foreign organisms. Overproduction of LTB_4 is involved in inflammatory diseases including psoriasis (5), bronchial asthma (6), rheumatoid arthritis (7), inflammatory bowel diseases (8), and ischemic renal failure (9). Mice lacking leukotriene production are deficient in their response to some acute and chronic inflammatory stimuli (10–13). Therefore, BLT antagonists are under development as potent antiinflammatory drugs (14–17). BLT antagonists have most recently been reevaluated as immunosuppressive agents for allograft rejection (18–20). We recently reported the characterization of cDNA encoding human BLT1 (originally termed BLT) isolated from HL-60 cells, and showed that BLT1 mRNA is highly expressed in leukocytes, and to a much lesser extent in the other tissues (21). BLT1s isolated from mouse (22, 23), guinea pig (24, 25) and rat (26) are highly homologous to hBLT1, with amino acid identities of >75% (27). During the course of the analysis of the genomic structures of human and mouse

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[1]*Abbreviations used in this paper:* ALXR, lipoxin A_4 receptor; CHO, Chinese hamster ovary; EST, expressed sequence tag; FPR, formyl peptide receptor; FPRL, formyl peptide receptor–like; GPCR, G protein–coupled receptor; HETE, hydroxyeicosatetraenoic acid; LTB_4, leukotriene B_4; LX, lipoxin; ORF, open reading frame; PTX, pertussis toxin; TM, transmembrane domain.

BLT1, we identified a novel gene encoding a putative GPCR with structural similarity to BLT1. Surprisingly, this receptor shows specific binding for LTB_4 and activates multiple intracellular signaling pathways when expressed in mammalian cells. In this study, we describe the molecular cloning of this novel LTB_4 receptor and demonstrate its specificity, expression, and function. Further, we show that the receptor has pharmacological properties different from those of BLT1. In an accompanying paper, we report that BLT2 open reading frame (ORF) is present in the promoter region of BLT1 (28), weaving these two receptors tightly at the genomic and functional level.

Materials and Methods

Isolation of Genomic Clones Containing Human and Mouse BLT1. Genomic libraries from human and mouse were screened by plaque hybridization. 10^6 clones from human genomic library (Human Lymphocyte Genomic Library; Stratagene), and mouse library (129SV Mouse Genomic Library; Stratagene) were lifted to Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) and screened with [^{32}P]dCTP-labeled ORF of hBLT1 and 800 bp of expressed sequence tag (EST) clones encoding mouse BLT1 (sequence data available from EMBL/GenBank/DDBJ under accession no. AA028322), respectively. Hybridization was carried out in a hybridization buffer containing 6× 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% SDS, followed by washing in 0.5× SSC, 0.1% SDS at 25°C. Tertiary screening gave three human and two mouse clones, which were analyzed by Southern blotting. DNA sequencing was done using an automated DNA sequencer (model 373A; Applied Biosystems) and LI-COR 4000LS (Aloka).

Northern Blotting. Human multiple-tissue Northern blots (CLONTECH Laboratories, Inc.) were hybridized with [^{32}P] dCTP-labeled ORF of hBLT2 and human β-actin cDNA in ExpressHyb hybridization solution (CLONTECH Laboratories, Inc.) for 18 h (BLT2) or 1 h (β-actin) at 68°C. The membranes were washed in 0.1× SSC, 0.1% SDS for 2 h at 65° C and subjected to autoradiography.

Construction of Expression Vectors for hBLT2. Two expression vectors for wild-type and hemagglutinin (HA)-tagged hBLT2 were constructed. The inserts were amplified from the genomic clone containing a full-length BLT2 by PCR with sense (5′-CGGGATCCCGCCATGTCGGTCTGCTACCGT-3′ and 5′-CGGGATCCCGCCATGTACCCCTACGACGTGCCCGACTACGCCTCGGTCTGCTACCGTCC-3′ for wild-type and HA, respectively) and antisense (5′-GGAATTCAAAGGTCCCATTCCGG-3′) primers, digested with BamHI and EcoRI, and subcloned into pcDNA3 vector (Invitrogen). The plasmids for wild-type and HA-tagged BLT2 were designated as phBLT2 and pHA-hBLT2, respectively. Entire sequences of the inserts were determined on both strands for unexpected misincorporations.

Cell Culture and Transfection. HEK 293 and Chinese hamster ovary (CHO) cells were cultured in DMEM and Ham's F12, respectively, supplemented with 10% FCS (Sigma-Aldrich), 100 IU/ml penicillin, and 100 μg/ml streptomycin. For transient expression, HEK 293 cells on 15-cm plates were transfected by lipofection using 20 μg of plasmid DNA and Lipofectamine Plus (Life Technologies) according to the manufacturer's protocol. After 3 d, the cells were harvested and sonicated in a sonication buffer containing 20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 10 mM $MgCl_2$, 2 mM EDTA-Na_2, 2 mM PMSF, 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 resulting pellets were used for binding assay as membrane fractions. The concentrations of the protein were determined by a method of Bradford using protein assay (Bio-Rad Laboratories). For stable expression of HA-tagged BLT2, CHO-K1 cells were transfected with pHA-hBLT2 by lipofection using Transfectam (Life Technologies) and selected with 1 mg/ml G418. 18 resistant clones were isolated by limiting dilution and examined for the expression of the receptor protein. The cells were fixed with PBS(−) containing 0.5% paraformaldehyde for 5 min on ice and blocked with PBS(−) containing 2% FCS. The cells were bound with 10 μg/ml anti-HA antibody (clone CA12-5; Eastman Kodak Co.) in PBS(−) containing 2% FCS for 1 h, followed by staining with 500× FITC-anti–mouse IgG (Zymed Laboratories) for 30 min. The cells were washed twice with PBS(−) and analyzed with a flow cytometer (Epics XL; Beckman Coulter). Three lines of the cells (HA-12, -13, and -14) with high expression were selected, maintained in Ham F12, 10% FCS, and 0.3 mg/ml G418, and used for the further analyses.

^{3}H–LTB_4 Binding Assay. The membrane fractions of HEK 293 cells were examined for ^{3}H–LTB_4 binding. The binding mixture (100 μl) contained membrane fractions and various concentrations of ^{3}H–LTB_4 with or without unlabeled LTB_4 in binding buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM NaCl, and 0.05% BSA). For determination of the nonspecific binding, at least 1,000 times concentration of unlabeled LTB_4 was used. The mixtures were incubated at room temperature for 60 min with agitation, followed by rapid filtration through GF/C filters (Packard Instrument Co.) and washing with ~3 ml of binding buffer. The radioactivities of the filters were determined with a scintillation counter (Top Count; Packard Instrument Co.).

Measurement of Intracellular Calcium Concentration. The CHO cells were loaded with 10 μM Fura-2 AM (Dojin) in Hepes-Tyrode's BSA buffer (25 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM d-glucose, 0.37 mM NaH_2PO_4, 0.49 mM $MgCl_2$, and 0.1% [wt/vol] fatty acid–free BSA; Fraction V) at 37°C for 2 h. The cells were washed twice and resuspended in Hepes-Tyrode's BSA buffer at the density of 10^6 cells/ml. 0.5 ml of the cell suspension was applied to a CAF-100 system (Jasco), and 5 μl of ligand solution in ethanol (for eicosanoids) or in PBS (for chemokines) was added. Intracellular Ca^{2+} concentration was measured by the ratio of emission fluorescence of 500 nm by excitation at 340 and 380 nm.

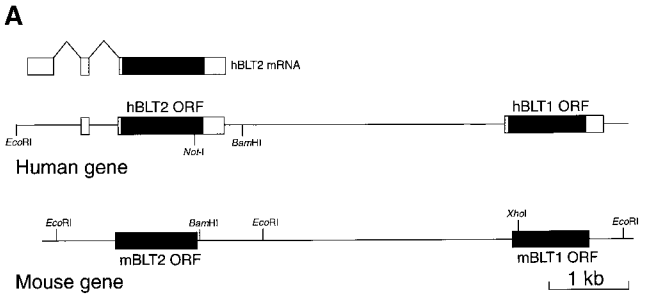

Figure 1. (*continues on facing page*).

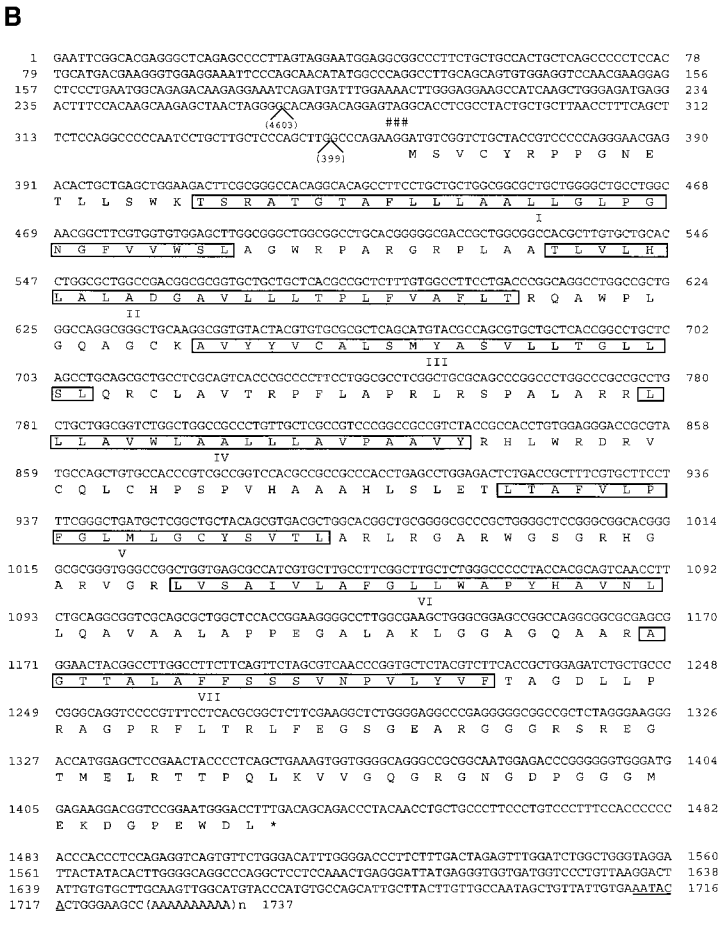

Figure 1. Cloning of human and mouse BLT2. (A) Structures of human and mouse genomic DNAs containing BLT1 and BLT2. In the human gene, transcribed segments are indicated by open boxes, and putative ORFs are indicated by filled boxes. (B) Primary structure of hBLT2 cDNA and deduced amino acid sequence. Putative TMs are boxed. An in-frame-stop codon (TAG) is indicated by number signs at the 5′ untranslated region of the putative ORF. The positions of introns are shown by the nucleotide numbers of introns. The incomplete polyadenylation signal (AATACA) is underlined. (C) Sequence alignment of BLT1 and BLT2 from humans and mice. Asterisks indicate amino acids conserved among four receptors. Dots indicate amino acids conserved among three receptors. The putative TMs of hBLT2 predicted from a Kyte-Doolittle hydrophobicity analysis are lined and labeled as I–VII. These sequence data are available from EMBL/GenBank/DDBJ under accession nos. AB029892 and AB029893.

Measurement of cAMP. The cells were seeded on 96-well plates (20,000 cells per well) and cultured for another 36 h. The medium was replaced to 100 μl of Hepes-Tyrode's BSA buffer containing 1 mM IBMX (3-isobutyl-1-methylxanthine), and incubated at 37°C for 20 min. The reaction was initiated by adding 100 μl of the ligand solution, and after 30 min of incubation, the reaction was terminated by adding 25 μl of lysis reagent 1A included in Biotrak cAMP enzyme immunoassay system (Amersham Pharmacia Biotech). The cAMP contents in 5 μl of the aliquots were determined using the assay kit according to the manufacturer's protocol.

Chemotaxis Assay. CHO cells expressing hBLT1 or hBLT2 were examined for their chemotactic responses as described previously (21).

Presentation of the Data. All figures shown contain representative data from at least two independent experiments with similar results.

Results

Cloning of Human and Mouse BLT2. During the course of the analysis of human and mouse BLT1 genes, we identified an ORF encoding a putative seven-transmembrane–type receptor with sequence similarities to BLT1. We designate the new gene BLT2. BLT1 and BLT2 genes are located within 10 kbp of each other both in humans and mice (Fig. 1 A). Because a NotI site is present in the ORF of hBLT2, we could not isolate full-length cDNA clones for hBLT2 from cDNA libraries (data not shown). However, we found several EST clones containing polyadenylation signals followed by poly A tails, or exon/intron junctions upstream of the ORF of hBLT2 (Fig. 1 B). The ORFs of human and mouse BLT2 encode proteins with 358 and 360 amino acids, respectively (Fig. 1 C), which contain several consensus GPCR sequences (29). These include 38N in the transmembrane domain (TM)-1; 38D in TM-2; 146W and 155P in TM-4; 237W, 239P, and 244N in TM-6; 284N, 285P, and 288Y in TM-7; and 94C and 168C, which may be joined by a disulfide bond. The amino acid identity between human and mouse BLT2 was 92.7%. A blast search showed that hBLT2 is highly homologous to human and mouse BLT1, with the amino acid identities of 45.2 and 44.6%, respectively. The similarity between BLT1 and BLT2 was high in the putative TMs, especially in TM-2, -3, and -7. Although BLT2 also showed significant homology to an orphan GPCR (GPR25 [30]) and a recently-cloned chemoattractant receptor–homologous molecule expressed on TH2 cells (CRTH2 [31, 32]), the homology was much lower (<31%) than that for BLT1.

Tissue Distribution of BLT2. Searching EST database revealed that BLT2 mRNA is expressed in various human tissues, including skeletal muscle, heart, lung, and mammalian gland. Northern blot analyses showed that BLT2 mRNA is expressed most abundantly in spleen, followed by liver, ovary, and leukocytes, with weak signals detected in most human tissues (Fig. 2). The size of the major transcript is 2.5 kb in these tissues, but longer transcripts are also detected. The results show that BLT2 expression is distinct from BLT1, which is expressed almost exclusively in leukocytes (21). BLT2 mRNA is also present in several human cell lines, including undifferentiated HL-60, K562, MOLT-4, SW-480, and A549 cells, but the size of these transcripts is much longer than those of the human tissues (Fig. 2).

Characterization of hBLT2 as a Pharmacologically Distinct LTB_4 Receptor. Because sequence similarities between BLT1 and BLT2 suggested that BLT2 is also a receptor for eicosanoids, we examined ^3H–LTB_4 binding to the membrane fractions of HEK 293 cells transiently transfected with an hBLT2 expression construct. We chose HEK 293 cells for transfection because they showed no specific binding for ^3H–LTB_4 at concentrations of up to 5 nM (data not shown). Membrane fractions of HEK 293 cells expressing hBLT2 showed a specific and saturable LTB_4 binding, whereas those of empty vector-transfected cells did not (Fig. 3 A). Scatchard analysis revealed that the Kd value of hBLT2 for LTB_4 was 22.7 nM (Fig. 3, B and C), which was 20-fold higher than that of hBLT1 (1.1 nM; data not

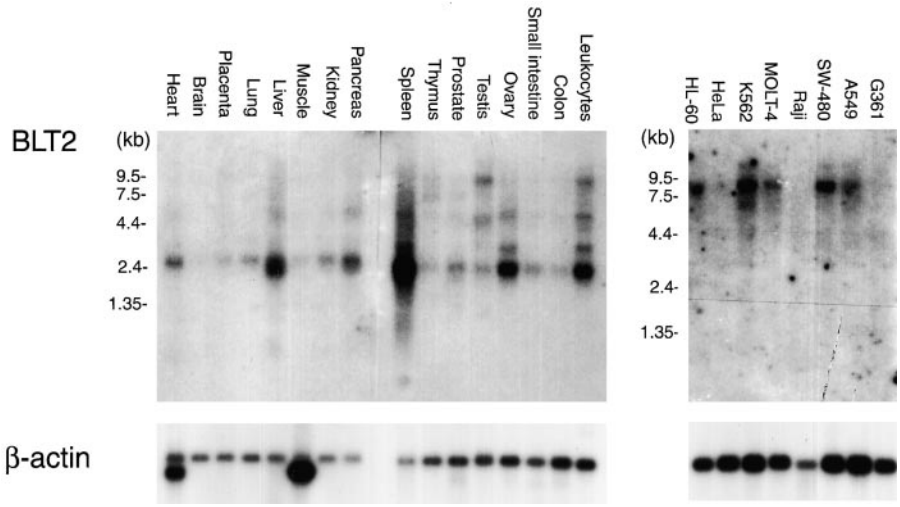

Figure 2. Northern blot analyses of BLT2 mRNA in various human tissues and cells. Human multiple-tissue Northern blot filters (2 μg poly-A RNA/lane; CLONTECH Laboratories, Inc.) were hybridized with [^{32}P]dCTP-labeled ORF of hBLT2 or human β-actin cDNA (CLONTECH Laboratories, Inc.).

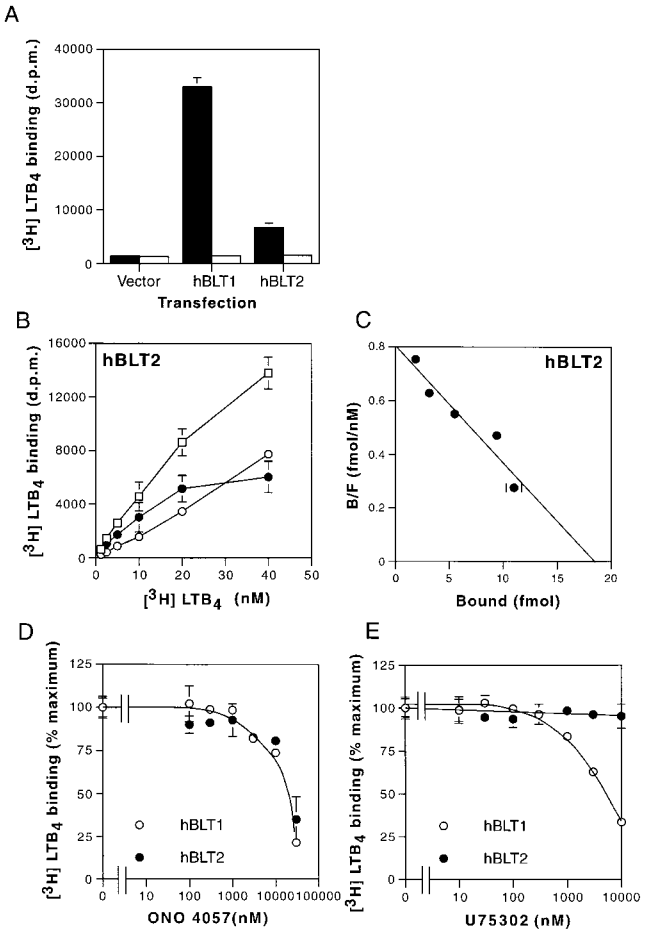

Figure 3. Binding of ^{3}H–LTB$_4$ to the membrane fractions of HEK 293 cells transiently transfected with hBLT1 or hBLT2. (A) Binding of 5 nM ^{3}H–LTB$_4$ to the membrane fractions (20 μg of protein) from HEK 293 cells transfected with control vector (Vector), BLT1 expression vector (hBLT1), or BLT2 expression vector (hBLT2). Total binding (black columns) and nonspecific binding (white columns) are presented (mean ± SD, n = 3). d.p.m., disintegration per minute. (B and C) Binding isotherms (B) and Scatchard analysis (C) of ^{3}H–LTB$_4$ binding to membrane fractions of HEK 293 cells transfected with hBLT2. In B, total binding (□), nonspecific binding (○), and specific binding (●) are presented (mean ± SD, n = 3). (D and E) Inhibition of 5 nM ^{3}H–LTB$_4$ binding to the membrane fractions (20 μg of protein) of HEK 293 cells transfected with hBLT2 (●) or hBLT1 (○) by two BLT antagonists, (F) ONO 4057 and (G) U 75302 (mean ± SD, n = 3).

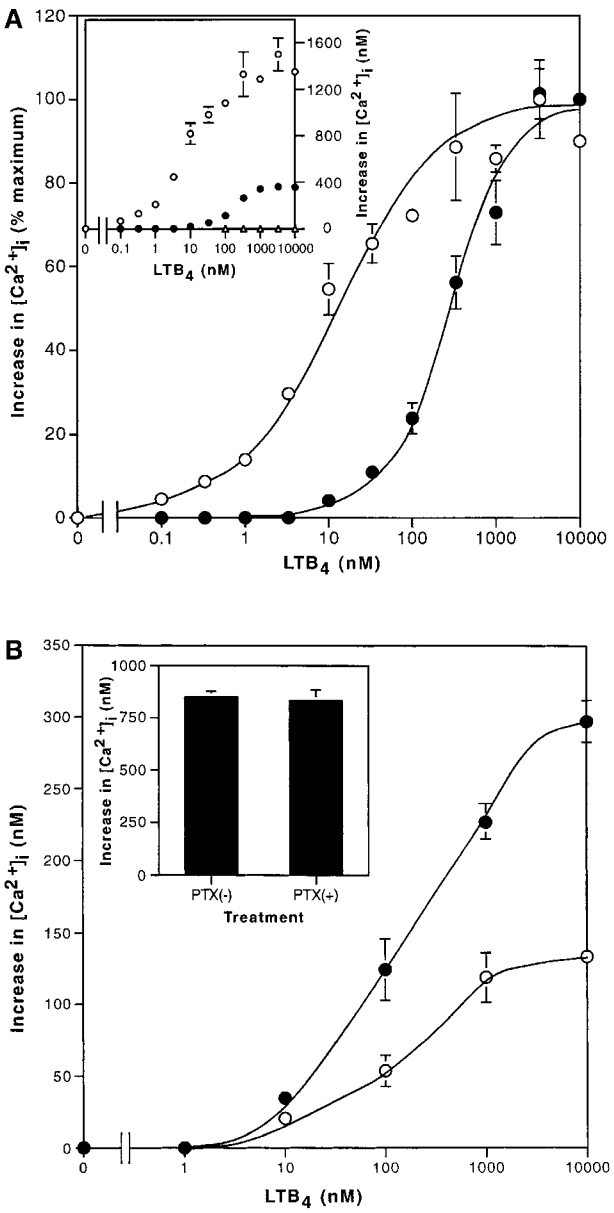

Figure 4. Calcium mobilization in CHO-hBLT1 and CHO-hBLT2 cells by LTB$_4$. (A) Increases in intracellular calcium after exposure to various concentrations of LTB$_4$ were measured in CHO-hBLT1 (○) and CHO-hBLT2 (●) cells, and were represented as percentages of the maximum responses. The inset graph shows absolute values of increase in intracellular calcium concentrations (mean ± SD, n = 3). (B) Effects of PTX pretreatment on LTB$_4$-induced increases in intracellular calcium concentrations in CHO-hBLT2 cells. The cells were pretreated with 100 ng/ml PTX (○) or vehicle (●) for 12 h. The inset shows increases in intracellular calcium concentrations, evoked by 2 U/ml thrombin, that were not affected by PTX pretreatment (mean ± SD, n = 3).

shown). We next examined the inhibition of ^{3}H–LTB$_4$ binding by two distinct BLT1 antagonists and various eicosanoids, using the membrane fractions of HEK 293 cells transfected with hBLT1 or hBLT2. Although ^{3}H–LTB$_4$ binding to hBLT1 was inhibited by both ONO 4057 and U 75302, ^{3}H–LTB$_4$ binding to hBLT2 was not inhibited by U 75302 (Fig. 3, D and E). Several other BLT antagonists also failed to inhibit LTB$_4$ binding to BLT2 (data not shown). ^{3}H–LTB$_4$ binding to BLT2 was competed with 5 μM LTB$_4$ (100%) or 20-hydroxy LTB$_4$ (78.6 ± 4.4% compared with the competition by LTB$_4$). 20-carboxy-LTB$_4$, lipoxin (LX)A$_4$, LXB$_4$, or 5-oxo-eicosatetraenoic acid slightly (<40%) competes with 5 nM ^{3}H–LTB$_4$ binding to BLT2 at the concentration of 5 μM. These results clearly show that BLT2 is a pharmacologically distinct receptor from BLT1 (21).

Intracellular Signaling of BLT2. To examine whether the binding of LTB$_4$ to BLT2 transduces intracellular signaling,

we established several lines of CHO cells stably expressing BLT2 (CHO-hBLT2) and compared LTB_4-induced cellular effects to those obtained with CHO cells expressing hBLT1 (CHO-hBLT1 [21]). CHO-K1 cells were transfected with an expression vector for HA-tagged hBLT2, selected with G418, and examined for expression of the tagged receptor using an anti-HA antibody. 18 clones were picked by limiting dilution, and 3 representative clones with high HA expression were selected (data not shown). As each of these three clones showed LTB_4-induced calcium mobilization and chemotaxis, we chose one clone as a representative and analyzed the LTB_4-induced intracellular signaling in more detail.

Fig. 4 A shows the increases in intracellular calcium concentrations induced by LTB_4 in CHO-hBLT1 and CHO-hBLT2 cells. In both cells, LTB_4 increased intracellular calcium in a dose-dependent manner, but the maximum increase in CHO-hBLT2 cells was only one third of that in CHO-hBLT1 cells (Fig. 4 A, inset). The dose response curve for LTB_4 in CHO-hBLT2 cells was right shifted from that in CHO-hBLT1 cells by 2 orders of magnitude (Fig. 4 A). Other ligands, including leukotriene C_4, leukotriene D_4, leukotriene E_4, 5(*S*)-hydroxyeicosatetraenoic acid (HETE), 5(*R*)-HETE, 12(*S*)-HETE, 12(*R*)-HETE, 15(*S*)-HETE, 15(*R*)-HETE, 5-oxo-eicosatetraenoic acid, LXA_4, LXB_4, IL-8, C5a (a component of complement), and FMLP, were not able to induce any significant change in intracellular calcium in CHO-hBLT2 cells at the concentrations of up to 1 μM (data not shown). 20-hydroxy-LTB_4, which partially inhibited ^3H–LTB_4 binding to BLT2, did not induce any change in intracellular calcium in CHO-hBLT2 at 1 μM (data not shown). To determine the subtypes of G protein(s) responsible for calcium increase by BLT2, we pretreated CHO-hBLT2 cells with 100 ng/ml pertussis toxin (PTX) for 12 h and examined the intracellular calcium response. Pretreatment of the cells with PTX diminished by half the response in CHO-hBLT2 cells (Fig. 4 B). PTX pretreatment of these cells did not affect the intracellular calcium increases induced with 2 U/ml thrombin (Fig. 4 B, inset), but completely abolished LTB_4-induced cell migration (see Fig. 6 B) in CHO-hBLT2 cells. Therefore, we conclude that the calcium response mediated by hBLT2 involves both PTX-sensitive and -insensitive G protein(s) in CHO cells.

We next examined the effects of LTB_4 stimulation on adenylyl cyclase activity in these cells by measuring the cAMP accumulation. In the absence of forskolin, which activates adenylyl cyclases, no increase in cAMP concentration was observed either in CHO-hBLT1 or CHO-hBLT2 cells (data not shown). On the other hand, LTB_4 inhibited 50 μM forskolin-activated adenylyl cyclase activities in a dose-dependent manner in both CHO-hBLT1 and CHO-hBLT2 cells. The IC_{50} value of LTB_4 for inhibiting adenylyl cyclase activities in CHO-hBLT2 cells (80 nM; Fig. 5 A) was higher than that of CHO-hBLT1 cells (0.1 nM; Fig. 5 A). Pretreatment of the cells with 100 ng/ml PTX for 12 h had markedly different effects on LTB_4-induced adenylyl cyclase inhibition. Although PTX pretreatment abolished ~80% of LTB_4-induced adenylyl cyclase inhibition in CHO-hBLT1 cells, the same pretreatment had a negligible effect on cAMP accumulation in CHO-hBLT2 cells (Fig. 5, B and C). These results indicate that BLT2 uses Gz, a PTX-insensitive G protein (33, 34), in inhibiting adenylate cyclases.

LTB_4 is a potent chemoattractant of granulocytes and macrophages, and CHO cells migrate toward LTB_4 when transfected with BLT1 (21). Examining the chemotactic activities of LTB_4 mediated by BLT2 using CHO-hBLT2

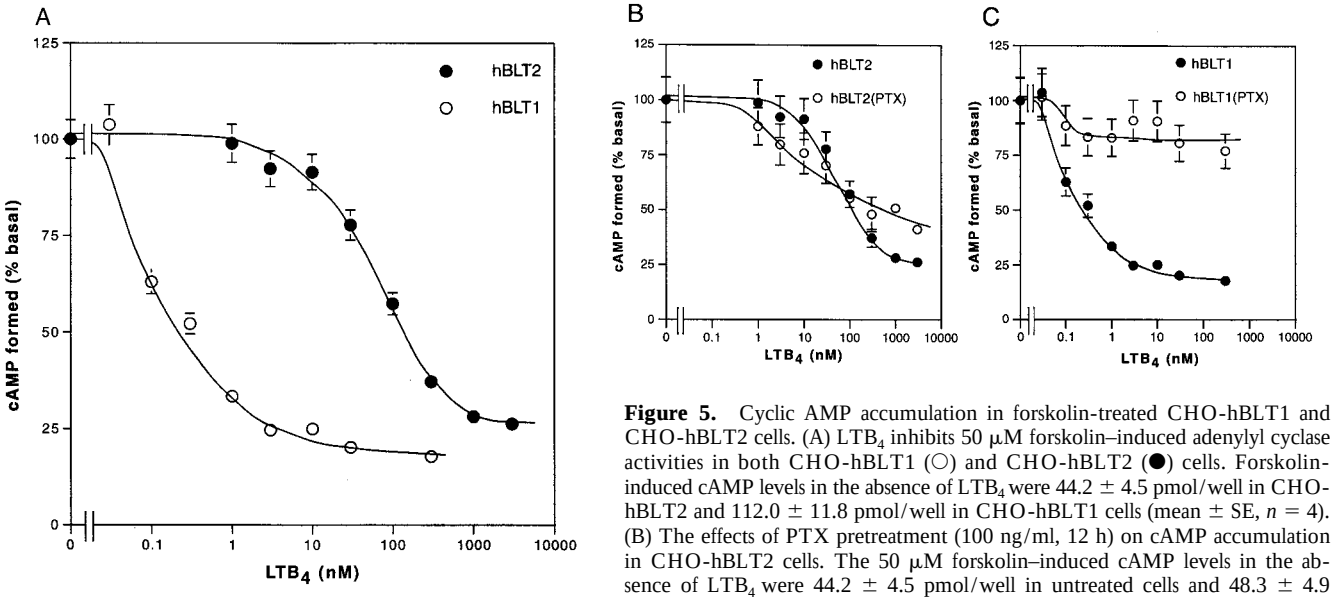

Figure 5. Cyclic AMP accumulation in forskolin-treated CHO-hBLT1 and CHO-hBLT2 cells. (A) LTB_4 inhibits 50 μM forskolin–induced adenylyl cyclase activities in both CHO-hBLT1 (○) and CHO-hBLT2 (●) cells. Forskolin-induced cAMP levels in the absence of LTB_4 were 44.2 ± 4.5 pmol/well in CHO-hBLT2 and 112.0 ± 11.8 pmol/well in CHO-hBLT1 cells (mean ± SE, $n = 4$). (B) The effects of PTX pretreatment (100 ng/ml, 12 h) on cAMP accumulation in CHO-hBLT2 cells. The 50 μM forskolin–induced cAMP levels in the absence of LTB_4 were 44.2 ± 4.5 pmol/well in untreated cells and 48.3 ± 4.9 pmol/well in PTX-treated cells (mean ± SE, $n = 4$). (C) The effects of PTX pretreatment (100 ng/ml, 12 h) on cAMP accumulation in CHO-hBLT1 cells. The 50 μM forskolin–induced cAMP levels in the absence of LTB_4 were 112.0 ± 11.8 pmol/well in untreated cells and 71.0 ± 7.3 pmol/well in PTX-treated cells (mean ± SE, $n = 4$).

cells, both CHO-hBLT1 and CHO-hBLT2 cells showed potent chemotactic activities, with bell-shaped dose response curves (Fig. 6 A). The optimum concentration of LTB_4 needed for chemotaxis in CHO-hBLT2 cells was higher than that of CHO-hBLT1 cells by 2 orders of magnitude, enhancing further the idea that hBLT2 is a low-affinity but still potent chemotactic receptor for LTB_4. As shown in Fig. 6 B, chemotaxis of CHO-hBLT2 cells induced by LTB_4 was abolished by PTX, showing that PTX-sensitive G protein(s) are absolutely required for LTB_4-induced cell migration through BLT1 and BLT2.

Discussion

LTB_4 is one of the most potent chemoattractants for leukocytes, and is unique because it is a lipid mediator biosynthesized from membrane phospholipids by the actions of cytosolic phospholipase A_2 (35, 36), 5-lipoxygenase, and LTA_4 hydrolase (3, 37). LTB_4–BLT interaction plays important roles in host defense mechanism and inflammatory diseases. Mice lacking in leukotriene production are insensitive to some inflammatory stimuli, and mice overexpressing BLT1 exhibit enhanced responses to infections and lung ischemic-reperfusion injury (38). Numerous biochemical and pharmacological studies indicated that high-affinity and LTB_4-specific receptors exist in membranes of neutrophils, macrophages, eosinophils, and T cells. Although many antagonists for LTB_4 receptor are under development, their main target has been directed to the high-affinity receptor for LTB_4, BLT1. There are also reports of low-affinity binding protein(s) for LTB_4 in human granulocytes (39), murine spleen (15), and guinea pig alveolar and peritoneal eosinophils (40, 41). Some investigators have speculated that the high-affinity receptor mediates chemotaxis for LTB_4, and the low-affinity receptor mediates LTB_4-induced secretary and oxidase-activation responses (42). Therefore, we have had only suggestive information on a low-affinity LTB_4 receptor.

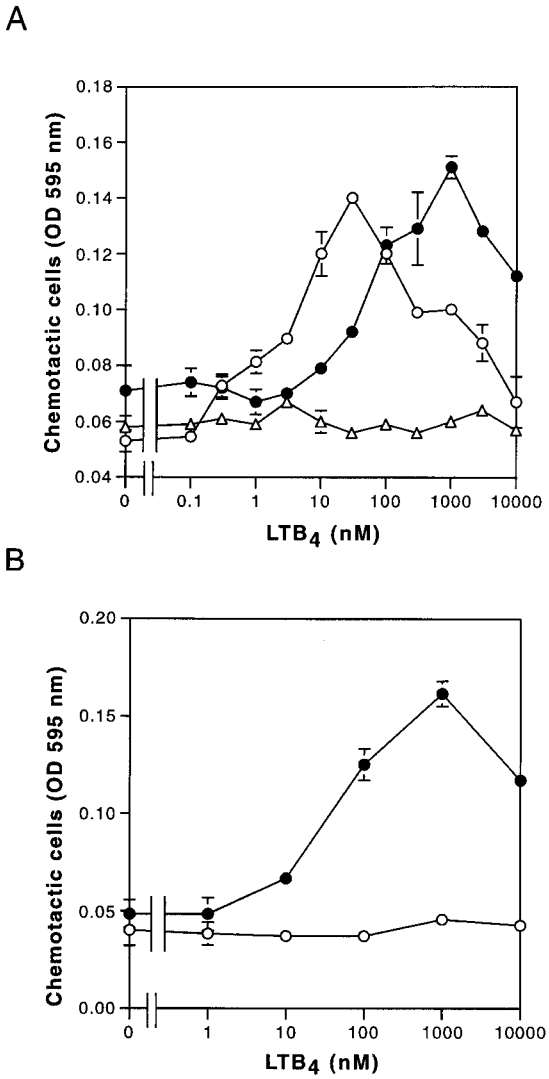

Figure 6. LTB_4-induced cell migration in CHO-hBLT1 and CHO-hBLT2 cells. (A) Dose dependency of LTB_4-induced cell migration was measured in CHO-hBLT1 (○), CHO-hBLT2 (●), and CHO vector (△) cells (mean ± SE, $n = 4$). (B) Effects of PTX pretreatment (100 ng/ml, 12 h) on LTB_4-induced cell migration in CHO-hBLT2 cells (mean ± SE, $n = 4$).

Identification of a Novel GPCR, BLT2, in a Gene Cluster with BLT1. Our interest in understanding the molecular mechanisms that regulate the transcription of BLT1 (originally termed BLT) gene led us to isolate several genomic clones containing BLT1 gene from humans and mice. The precise structure of the hBLT1 gene and the mechanism by which the BLT1 gene is regulated is published in an accompanying paper (28). During the course determining the nucleotide sequences of these genomic clones, we discovered an ORF for a putative seven-transmembrane receptor (BLT2) that is similar to BLT1 (Fig. 1, A and B). Human and mouse BLT2 genes encode proteins of 358 and 360 amino acids, respectively (Fig. 1 C), similar to hBLT1 (352 amino acids). The amino acid identity of hBLT1 and hBLT2 is 45.2%, and that of human and mouse BLT2 is 92.7%. The amino acid identity of BLT2 between human and mouse is higher than BLT1 between two species (78.6%), suggesting that BLT2 has been conserved during evolution and must therefore play an important role. We conclude that BLT2 is not a pseudogene based on the following criteria: (a) Northern blotting showed that mRNA expression is seen in various tissues and cells in humans; (b) the primary structures of human and mouse BLT2 are well conserved, with the amino acid identity of 92.7%; (c) the structures of genomic clones containing BLT1 and BLT2 are similar both in humans and mice; (d) there are no frame shifts in either human or mouse BLT2 ORF; and (e) many EST clones encoding BLT2 have been deposited in the EMBL/GenBank/DDBJ database. hBLT2 showed a significant homology to a recently cloned chemoattractant receptor–homologous molecule expressed on TH2 cells (31, 32) and an orphan GPCR, GPR25 (30), with amino acid identities of 30.5 and 28.5%, respectively. A recently cloned receptor for cysyteinyl leukotrienes, Cys-LT1, showed 28% amino acid identity to hBLT1 (43, 44). Thus, BLT2 is the second member for a LTB_4 receptor family.

hBLT1 was reported to be located on human chromosome 14q11.2-q12 (45), and our present finding shows that the ORF for hBLT2 is located at 3 kbp upstream of hBLT1 ORF (Fig. 1 A). Similar gene clusters of GPCRs have been reported for the FMLP receptor, its related receptors (46), and C5a receptor on chromosome 19q13.3-q13.4 (47), and for CXCR1 and 2, the receptors for IL-8, on 2q34-q35 (48–50). β2 and α1 adrenergic receptors are also closely linked on human chromosome 5q32-q34, and β1 and α2 adrenergic receptors are both located on human chromosome 10q24-q26 (51). Formyl peptide receptor (FPR)-like (FPRL) 1–LXA_4 receptor (AXLR), which is a neighboring receptor to the FMLP specific receptor (FPR1), was reported to respond to lipoxin A_4 at nM (52) and the acute phase protein serum amyloid A at μM (53). It was shown recently that MHC binding peptide and synthetic peptide MMK-1 bind to FPRL1/ALXR at sub-μM concentration (54). In the case of IL-8 receptors, CXCR1 is a high-affinity and specific receptor for IL-8, and CXCR2 is a less selective receptor for IL-8, which also binds growth regulatory oncogene (GRO)-α, -β, -γ and neutrophil-activating peptide (NAP)-2 (55). These receptor clusters appear to be generated by gene duplication, with the degree of homology among the members of the cluster indicative of the timing of the duplication during evolution (50). The amino acid identity between hBLT1 and 2 (45.2%) is much lower than that between CXCR1 and 2 (77%) and between FPR1 and FPRL1/ALXR (72%), suggesting that the duplication event that generated BLT2 occurred earlier than the events generating the other receptor clusters. The reasons why these receptors for chemoattractants form clusters are not yet clear, but may become evident as more information is gathered. Surprisingly, the promoter region of BLT1 is localized in the ORF of BLT2. This, to our knowledge, is the first mammalian example of "promoter in ORF," which is reported in bacteria (56) and bacteriophages (57).

Characterization of BLT2 as a Low-Affinity Receptor for LTB_4. We first examined whether or not BLT2 can recognize LTB_4. A binding assay using membrane fractions from HEK 293 cells exogenously transfected with the receptor cDNA showed that BLT2 is a low-affinity receptor for LTB_4 (Fig. 3, A–E). The Kd value was 22.7 nM, which is higher than that of BLT1 (21–26). High- and low-affinity LTB_4 binding sites were reported in human (42, 58) and rabbit (59) neutrophils, differentiated HL-60 cells (60, 61), guinea pig peritoneal eosinophils (41), and murine spleen membrane (62). The Kd values of these low-affinity binding sites for LTB_4 were reported to be between 70 and 580 nM, similar to the value obtained from the transfection study using BLT2 (Fig. 3, B and C). Thus, BLT2 appears to be a low-affinity binding site for LTB_4. The high expression of BLT2 in spleen on Northern blots (Fig. 2) also supports this speculation, as spleen is the tissue where the low-affinity receptor is most well characterized (14, 15, 62). Studies using BLT antagonists showed that BLT2 is a pharmacologically novel receptor for LTB_4, as binding of LTB4 to BLT2 was not inhibited by a classical BLT antagonist, U 75302 (references 21, 63; Fig. 3, D and E).

Signaling from BLT2. We next established several lines of CHO cells stably expressing BLT2 to examine intracellular signaling. CHO cells were transfected with an expression construct for HA-tagged BLT2 and were selected with culture media containing G418, and stable clones were isolated. All three cell lines selected for high HA expression responded to LTB_4 with calcium mobilization and chemotaxis, as opposed to cells transfected with the empty vector, which did not respond. In calcium mobilization assays, IC_{50} values for LTB_4 in CHO-hBLT1 and CHO-hBLT2 cells were 10 nM and 300 nM, respectively (Fig. 4 A). Calcium increases in CHO-hBLT1 cells were not inhibited by PTX pretreatment (21), and cotransfection of BLT1 cDNA with Gαq, but not with Gα11, increased LTB_4-induced d-myo-inositol 1, 4, 5-triphosphate (IP_3) accumulation in Cos-7 cells (4). LTB_4–BLT1 interaction appears to activate Gαq protein, leading to the activation of phospholipase C. On the other hand, about half of the calcium increase by LTB_4 through BLT2 was inhibited by pretreatment of PTX (Fig. 4 B), suggesting that different types and/or different ratios of G proteins are activated by binding of LTB_4 to BLT2. Differences in the maximum responses in LTB_4-induced calcium mobilizations in CHO-BLT1 and CHO-BLT2 cells (Fig. 4 A, inset) also support this hypothesis. As the low-affinity binding of LTB_4 to BLT2 raised the possibility of the other ligands for this receptor, we examined calcium mobilization in CHO-BLT2 cells by various eicosanoids and chemoattractants. Other than LTB_4 (discussed in Results), none of the ligands tested showed positive responses up to 1 μM, whereas 100 nM LTB_4 showed a clear calcium increase (Fig. 4).

With respect to inhibition of adenylyl cyclase, PTX pretreatment had different effects on these two related receptors (Fig. 5 A). LTB_4-mediated inhibition of adenylyl cyclase in CHO-hBLT1 cells is completely PTX sensitive, but in CHO-hBLT2 it is hardly affected by PTX pretreatment (Fig. 5, B and C), suggesting that BLT1 and BLT2 use different subtype(s) of Gi-like G proteins. Among Gi family members with inhibitory effects on adenylyl cyclases, only Gzα is insensitive to PTX, as it lacks the Cys residue at position (−4) from the COOH terminus (33, 34). Therefore, BLT2 may use Gzα to inhibit adenylyl cyclase in transfected CHO cells. On the other hand, LTB_4-induced chemotactic activities of CHO-hBLT1 and CHO-hBLT2 cells were completely PTX sensitive (Fig. 5 B). Thus BLT2, like BLT1, uses at least three classes of G proteins (Gi, Gq-like, and Gz) for signaling.

BLT2, a Possible Therapeutic Target. A unique characteristic of BLT2 is its ubiquitous expression, with the highest mRNA levels in spleen, followed by liver, ovary, and peripheral leukocytes (Fig. 2). In ovary, LTB_4 was reported to mediate ovulation in rat (64) and rabbit (65), without affecting the production of estradiol and progesterone from the ovarian cells. The biological roles of LTB_4 in liver are unknown, but the liver expresses the LTB_4-producing enzyme LTA_4 hydrolase (66, 67) and LTB_4-degrading enzymes LTB_4 20-hydroxylase (68, 69), LTB_4 12-hydroxydehydrogenase (70), and β-oxidizing enzymes (71). The liver

also expresses high amounts of peroxisome proliferator-activated factor (PPAR)α, a reported nuclear receptor for LTB_4, which controls transcription of various genes involved in lipid metabolism (72, 73). Therefore, it is reasonable to consider that LTB_4 has some as yet unidentified roles in the liver function that are mediated by BLT2. Northern blotting shows that human peripheral leukocytes express both BLT1 and BLT2 (Fig. 2; reference 21), which agrees well with a previous report of the coexistence of high- and low-affinity LTB_4 receptors in these cells (39). Some investigators have speculated that the high-affinity receptor mediates chemotaxis for LTB_4 and the low-affinity receptor mediates LTB_4-induced degranulation and superoxide anion generation (42). Transfection studies using BLT1 or BLT2 or both will enable us to precisely analyze and distinguish the roles of two receptors in vitro. We should again emphasize that LTB_4 binding to BLT2 is not inhibited by most of the previously developed BLT antagonists, suggesting that BLT2 could be a therapeutic target for novel drugs related to contraception and immunosuppression. The lack of significant effects of the current BLT antagonists in some studies may be due to the lack of their effects on BLT2. Further study will be needed to clarify the biological roles of LTB_4 through BLT2, especially in relation to the spleen, and in ovaries and the liver. Although LTB_4 was the best ligand among those tested for hBLT2, we should pay attention to the possible existence of better ligand(s) for BLT2 than LTB_4.

In conclusion, we have identified a second leukotriene B_4 receptor, BLT2, from humans and mice. The BLT2 gene closely located to the BLT1 gene in both humans and mice. In an accompanying paper (28), we detail the presence of the ORF for BLT2 localized in the promoter region of BLT1. BLT2 is a low-affinity and pharmacologically novel receptor for LTB_4, activating different G protein(s) to increase intracellular calcium, inhibit adenylyl cyclase, and stimulate chemotaxis. These findings may lead us to identify other members of LTB_4 receptor family, and provide new important clues concerning the physiological and pathophysiological roles of LTB_4.

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