cDNA cloning and characterization of guinea-pig leukotriene B_4 receptor

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The cDNA for leukotriene B_4 (LTB_4) receptor (BLT) was cloned from a guinea-pig leucocyte cDNA library. The cloned receptor cDNA encodes 348 amino acid residues and shares 73 % identity with the amino acid sequence of human BLT. Northern blot analysis showed the highest expression of the receptor mRNA in leucocytes, followed by lung and spleen. The membrane fractions of HEK-293 and Cos-7 cells transfected with the cDNA showed specific LTB_4-binding activities, with K_d values of 0.27 and 0.17 nM respectively. *Xenopus laevis* oocytes injected with the cRNA of guinea-pig BLT showed LTB_4-induced Cl^- currents, indicating that the cloned receptor is functional. LTB_4 is metabolized to 20-hydroxy-LTB_4 and then to 20-carboxy-LTB_4, a transformation considered as a major inactivation pathway of the compound. Using the cloned receptor, we analysed the agonistic effects of LTB_4 and these two metabolites. 20-Carboxy-LTB_4 is a much weaker agonist, with a K_d value higher than that of LTB_4 by three orders of magnitude, corresponding to a much weaker chemotactic activity. Although 20-hydroxy-LTB_4 is as potent as LTB_4 in inhibiting [^{3}H]LTB_4 binding and cAMP formation, it is less potent than LTB_4 in the mobilization of intracellular Ca^{2+} and the chemotaxis of Chinese hamster ovary cells expressing the guinea-pig BLT. The present study demonstrated that although LTB_4 and 20-hydroxy-LTB_4 bind to the receptor with similar affinities, they do differ in activating intracellular signalling.

Key words: chemotaxis, G-protein-coupled receptor, 20-hydroxyleukotriene B_4, inflammation, leucocytes.

INTRODUCTION

Leukotriene B_4 [(5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; LTB_4] is a potent activator of leucocytes that is derived from arachidonic acid by the action of 5-lipoxygenase and leukotriene A_4 hydrolase [1]. LTB_4 is an inducer of chemotactic and chemokinetic migration and of the adhesion of polymorphonuclear leucocytes to endothelial cells [2–4]. It also activates polymorphonuclear leucocytes to generate superoxide anions [5] and to release lysosomal enzymes [6,7]. These biological responses induced by LTB_4 are important in host defence mechanisms against foreign organisms. The biological properties of LTB_4 are comparable to those of other chemotactic factors, such as C5a and bacterial formyl peptide ('fMLP'), and are considered to be mediated through a G-protein-coupled receptor expressed on the cell surface of leucocytes [8–10].

Recently we reported the isolation of cDNA for an LTB_4 receptor (BLT) from HL-60 human leukaemia cells with the use of a subtraction strategy [11]. Human BLT showed specific binding of LTB_4 with a K_d of 0.154 nM when expressed in Cos-7 cells. In Chinese hamster ovary (CHO) cells carrying human BLT, LTB_4 induced the accumulation of Ins(1,4,5)P_3, increased intracellular Ca^{2+} concentrations and caused a chemotactic response. cDNA for BLT was also cloned from murine eosinophils [12]. The higher expression of mRNA for BLT was detected in eosinophils from interleukin-5-transgenic mice, suggesting the possible role of interleukins in the transcriptional regulation of BLT. BLT was also reported to be a functional HIV-1 co-receptor in CD4-positive cells [13], like the other chemokine receptors CCR4 and CXCR5 [14]. The chemokines, in controlling leucocyte functions and trafficking, bind to the cell surface receptors, members of a G-protein-coupled receptor family [15]. In contrast with the overlapping biological properties with chemokines, human and murine BLTs showed low similarity to the other chemokine receptors, suggesting that the BLTs might constitute a novel receptor family.

LTB_4-specific binding has been characterized on guinea-pig lung membranes [16,17], spleen [18], pulmonary eosinophils [19] and macrophages [20]. The application *in vivo* of LTB_4 stimulates neutrophil adhesion in post-capillary venules, emigration into tissues, and increased vascular permeability. The guinea-pig is frequently used for the study of inflammatory responses by LTB_4 [21,22]. However, the primary structure and precise tissue distribution of guinea-pig BLT have not been presented.

We isolated BLT from guinea-pig to understand better the amino acids conserved between various species, which might be important for the recognition of LTB_4 and possibly HIV-1 as well as for the coupling to the intracellular signalling molecules. Here we report the primary structure, tissue distribution and functional expression of guinea-pig BLT in mammalian cells as well as in *Xenopus* oocytes. Particular attention was directed to receptor binding and effector activation by LTB_4 and its metabolites.

MATERIALS AND METHODS

cDNA cloning

The cDNA library in λZAPII vector (Stratagene, La Jolla, CA, U.S.A.) was constructed from guinea-pig leucocyte mRNA. Clones (5×10^5) were transferred to Hybond-N$^+$ nylon membranes (Amersham, Little Chalfont, Bucks., U.K.) and screened by hybridization with the [^{32}P]dCTP-labelled open reading frame (ORF) of human BLT [11]. The hybridization was performed at

Abbreviations used: BLT, leukotriene B_4 receptor; CHO, Chinese hamster ovary; HETE, hydroxy-5,8,14-*cis*,10-*trans*-eicosatetraenoic acid; LPA, lysophosphatidic acid; LTB_4, leukotriene B_4 [(5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid]; ORF, open reading frame; RP-HPLC, reverse-phase HPLC.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AB005049.

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37 °C in a hybridization buffer containing 5 × SSC (SSC being 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution (0.1 % Ficoll 400/0.1 % polyvinylpyrrolidone/0.1 % BSA), 0.2 % SDS and 50 μg/ml salmon sperm DNA, and the membranes were washed at 50 °C in a washing buffer containing 0.5 × SSC and 0.1 % SDS. After tertiary screening, 10 clones were isolated and the plasmids were recovered by excision *in vivo*. The cDNA inserts were sequenced with an ABI 373 DNA Sequencer (Perkin-Elmer, Norwalk, CT, U.S.A.). The sequencing showed that two clones of 1525 bp (mgpl-3) and 1336 bp (mgpl-2) contained an identical ORF for a seven-transmembrane receptor; the other clones encoded the partial cDNAs of these two clones.

Northern blot analysis

Poly(A)$^+$ RNA (6 μg) from various tissues of guinea pig were separated in a 1 % (w/v) agarose gel containing 2 % (v/v) formaldehyde, then transferred to a Hybond-N nylon membrane (Amersham). The membrane was hybridized with [^{32}P]dCTP-labelled ORF of the mgpl-3 clone or a human glyceraldehyde-3-phosphate dehydrogenase cDNA (Clontech, Palo Alto, CA, U.S.A.) in a hybridization buffer containing 4 × SSC, 5 × Denhardt's solution, 0.2 % SDS, 200 μg/ml salmon sperm DNA and 50 % (v/v) formamide at 42 °C for 24 h. The membrane was washed in a washing buffer containing 0.1 × SSC and 0.1 % SDS at 65 °C, then subjected to autoradiography.

Cell culture and transfection

The ORF of guinea-pig BLT was amplified by PCR with the primers (sense 5′-CGGGATCCTTGTGATGGACAGGAAC-3′, anti-sense 5′-CGGAATTCCGCCAGGCCTAGTCTGATT-CGC-3′). The resulting fragment was subcloned into *Bam*HI and *Eco*RI sites of pcDNA3 expression vector (Invitrogen, San Diego, CA, U.S.A.), designated pGPBR. The Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys ('FLAG')-tagged expression construct was prepared in the same way with a sense primer (5′-CGGGA-TCCCGATGGACTACAAGGACGACGATGACAAGGAC-AAGAACACTACAACTC-3′) and was designated pFGPBR. Entire ORFs were sequenced and did not contain any unexpected misincorporations. HEK-293 and Cos-7 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) containing 10 % (v/v) fetal calf serum (Sigma, St. Louis, MO, U.S.A.). These cells were transiently transfected with pGPBR or an empty vector by lipofection by using Transfectam (Biosepra, Marlborough, MA, U.S.A.) in accordance with the manufacturer's protocol and were cultured for 72 h before analysis. CHO cells were grown in Ham's/F-12 medium (Nipro, Osaka, Japan) with 10 % (v/v) fetal calf serum. CHO cells were transfected with pFGPBR or an empty plasmid by using Transfectam; stable transformants were selected with 1 mg/ml of G418 (Wako, Osaka, Japan) for 10 days. The cells were cloned by two rounds of serial dilution and were analysed for binding of [^{3}H]LTB_4. A representative clone, CHO-GPF10, with the highest binding activity, was maintained in 0.3 mg/ml G418, and was used for further analyses.

Radioligand binding assay

The cells were sonicated in homogenizing buffer [20 mM Tris/HCl (pH 7.4)/0.25 M sucrose/10 mM $MgCl_2$/1 mM EDTA/0.5 mM PMSF/2 mM dithiothreitol] with a sonicator Model 5202PZT (Otake, Tokyo, Japan). All procedures were performed at 0–4 °C unless stated otherwise. The sonicates were centrifuged at 13000 *g* for 15 min; the supernatants were further centrifuged at 130000 *g* for 1 h. The precipitates (the membrane fractions) were suspended in homogenizing buffer and stored at −20 °C until use. The [^{3}H]LTB_4 binding assay was performed in 200 μl of binding buffer [50 mM Tris/HCl (pH 7.4)/10 mM $MgCl_2$/10 mM NaCl/0.1 % fatty-acid-free BSA] containing the membrane fraction and [^{3}H]LTB_4 with or without competitors. The binding mixtures were incubated at room temperature for 1 h. The incubation was terminated by filtration of the assay mixtures through GF/C glass-fibre filters (Packard, Meriden, CT, U.S.A.). The filters were washed with approx. 3 ml of binding buffer and dried; the remaining radioactivities were measured in a Top-count microplate scintillation counter (Packard).

Reverse-phase HPLC analysis

To determine the purity of LTB_4 and to exclude potential conversion during the incubation of LTB_4 with membrane fractions, reverse-phase (RP) HPLC analyses were performed [23]. Solvents A and B contained methanol/acetonitrile/water/acetic acid (35:25:45:0.02, by vol.), and methanol/acetonitrile (75:25, v/v) respectively. LTB_4 (1 μM) was incubated in 200 μl of binding buffer with or without membrane fractions of transfected HEK-293 cells at room temperature for 1 h. The lipid fraction was extracted from the mixture by 200 μl of ethyl acetate; 150 μl of the aliquot was dried in a centrifugal concentrator (Sakuma, Tokyo, Japan). Solvent A (200 μl) was added; an aliquot (100 μl) was analysed by RP-HPLC. We used a Cosmosil (5D-18AR, 4.6 mm × 150 mm; Nakalai Tesque, Kyoto, Japan) RP column equipped with a Beckman System Gold liquid chromatography system with a diode-array-detector module model 168; the column was pre-equilibrated with solvent A. Samples were eluted at a flow rate of 1 ml/min with continuous monitoring of UV absorbance at 270 nm. At 5 min after injection of the sample, the column was eluted with a linear gradient of solvent B (10 %/min). After a further 6 min, the column was eluted isocratically with 40 % solvent A and 60 % solvent B for 12 min, then with a linear gradient of solvent B (20 %/min) to 100 %.

Expression of guinea-pig BLT in *Xenopus laevis* oocytes

pGPBR was linearized by *Not*I and transcribed to cRNA *in vitro* with MEGAscript (Ambion, Austin, TX, U.S.A.) in the presence of a cap analogue, 7-methyl-G(5′)ppp(5′)G (Ambion). Transcripts (25 ng) dissolved in 50 nl of sterile water were injected into defolliculated oocytes, which were incubated at 18 °C for a further 1–2 days. The membrane potential of oocytes was clamped at −50 mV in modified Ringer's solution [115 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/5 mM Hepes (pH 7.4)] containing 0.1 % fatty-acid-free BSA (Sigma), and the Cl^- current elicited by bath-applied LTB_4 was recorded by a voltage-clamp method [24].

Measurement of intracellular Ca^{2+} concentrations

The intracellular Ca^{2+} concentrations were measured basically as described [25]. CHO-GPF10 cells were loaded with 3 μM fura-2 acetoxymethyl ester (Dojin, Kumamoto, Japan) in Hepes/Tyrode's/BSA buffer [140 mM NaCl/2.7 mM KCl/1.8 mM $CaCl_2$/0.49 mM $MgCl_2$/12 mM $NaHCO_3$/0.37 mM NaH_2PO_4/5.6 mM D-glucose/2.5 mM Hepes (pH 7.4), containing 0.1 % fatty-acid-free BSA] for 2 h at 37 °C. After loading, the cells were washed and resuspended in Hepes/Tyrode's/BSA buffer to a concentration of 2×10^6 cells/ml. Ligands were applied and

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changes in intracellular Ca^{2+} concentrations were measured with a Ca^{2+} analyser CAF100 system (Jasco, Tokyo, Japan).

Measurement of intracellular cAMP concentrations

CHO-GPF10 cells were incubated for 20 min in Hepes/Tyrode's/BSA buffer containing 0.5 mM 3-isobutyl-1-methylxanthine at 37 °C. The cells were exposed to 50 μM forskolin in the presence of eicosanoids for 20 min. The reactions were terminated by adding ice-cold $HClO_4$ to a final concentration of 6 % (w/v). After centrifugation, the supernatants were neutralized to pH 7.4, and the cAMP concentrations were measured with a cAMP radioimmunoassay kit (Yamasa, Chiba, Japan) in accordance with the manufacturer's protocol.

Chemotaxis assay

Measurements of chemotactic activities of CHO-GPF10 cells towards eicosanoids were performed with the use of an established protocol [11]. Framed polycarbonate filters with 8 μm pores (Neuroprobe, Cabin John, MA, U.S.A.) were coated with 10 μg/ml fibronectin (Wako) in PBS and placed in a NeuroProbe 96-well chemotaxis chamber. The lower blind wells were filled with various eicosanoids in Ham's/F-12 medium containing 0.1 % BSA. CHO-GPF10 cells were suspended in Ham's/F-12 medium containing 0.1 % BSA at a density of 2.5×10^5 cells/ml; 200 μl aliquots were applied to the upper wells of the chemotaxis chamber. After incubation for 3.5 h at 37 °C, the filters were stained with the use of a Diff-Quick staining kit (International Reagents Corp., Kobe, Japan) and were scraped free of cells on the upper side. The cell migrations to the lower sides of the filters were quantified by measurement of A_{595}.

RESULTS

A guinea-pig leucocyte cDNA library was screened by low-stringency hybridization with the ORF of human BLT as a probe. Ten clones were isolated and subjected to DNA sequence analyses. Two clones, termed mgpl-2 and mgpl-3, containing 1336 bp and 1525 bp, respectively, encoded the same putative ORF for a seven-transmembrane receptor. The ORF of mgpl-2 and mgpl-3 was 1044 bp in length, encoding a protein of 348 amino acid residues, which was shorter than human BLT by four residues. Figure 1 shows the amino acid alignment of guinea-pig, human [11] and murine [12] BLT. Guinea-pig BLT has 73 % and 70 % amino acid identity with human and murine BLT respectively. The identity of the putative transmembrane domains between guinea-pig and human BLT was 86 %. In addition, BLTs of these three species have the two conserved potential N-glycosylation sites (Asn-4 and Asn-165 in guinea-pig BLT) at the N-terminus and in the third extracellular region. The two cysteine residues in the second and the third extracellular loops were also conserved in guinea-pig, human and murine BLT. The Asp-Arg-Ser (DRS) sequence in the second intracellular loop corresponded to the conserved Asp-Arg-Tyr (DRY) sequence in other G-protein-coupled receptors [26,27]; the clustered basic amino acids in the third intracellular loop of human and murine BLT were also conserved in guinea-pig BLT. It contained five potential phosphorylation sites for protein kinase C, one site in each of the second and third intracellular loops (Ser-127 and Thr-221 in guinea-pig BLT) and three in the C-terminal tail (Ser-313, Thr-314 and Thr-323 in guinea-pig BLT). Several serine and threonine residues located in the same regions constitute targets for the family of G-protein-coupled receptor kinases [28] and could be involved in the agonist-dependent desensitization of the receptor [29].

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guinea pig   1:MDRNTTTRAASPSGSNTFIPLLAMILLSVSMVVGLPGNTFVVWSILKRMRKRSVTALMVL  60
human        1:M--NTTSSAAPPSLGVEFISLLAIILLSVALAVGLPGNSFVVWSILKRMQKRSVTALMVL  58
mouse        1:MAANTTSPAAPSSPGGMSLSLLPIVLLSVALAVGLPGNSFVVWSILKRMQKRTVTALIVL  60
                                          TM1

guinea pig  61:NLALADLAVLLTAPFFLHFLTWHTWSFKLAGCRLCHYICGVSMYASVLLITAMSLDRSLA 120
human       59:NLALADLAVLLTAPFFLHFLAQGTWSFGLAGCRLCHYVCGVSMYASVLLITAMSLDRSLA 118
mouse       61:NLALADLAVLLTAPFFLHFLARGTWSFREMGCRLCHYVCGISMYASVLLITIMSLDRSLA 120
                 TM2                                   TM3

guinea pig 121:VASPFLSQKVRTKTAARWLLVGIWGASFLLATPVLAFRKVVKLTNETDLCLAVYPSDRHK 180
human      119:VARPFVSQKLRTKAMARRVLAGIWVLSFLLATPVLAYRTVVPWKTNMSLCFPRYPSEGHR 178
mouse      121:VARPFMSQKVRTKAFARWVLAGIWVVSFLLAIPVLVYRT-MKWNNRTLICAPNYPNKEHK 179
                                      TM4

guinea pig 181:AFHLLFEAFTGFVVPFLIVVASYADISRRLRVRRFHRRRRTGRLVVIIILAFAAFWLPYH 240
human      179:AFHLIFEAVTGFLLPFLAVVASYSDIGRRLQARRFRRSRRTGRLVVLIILTFAAFWLPYH 238
mouse      180:VFHLLFEAITGFLLPFLAVVASYSDIGRRLQARRFRRSRRTGRLVVLIILAFAAFWLPYH 239
                         TM5                                         TM6

guinea pig 241:VVDLVEGSRVLAG---TLDQSKQQLRNARKVCIALAFLSSSVNPLLYACAGGGLLRSAGV 297
human      239:VVNLAEAGRALAGQAAGLGLVGKRLSLARNVLIALAFLSSSVNPVLYACAGGGLLRSAGV 298
mouse      240:LVNLVEAGRTVAGWDKN-SPAGQRLRLARYVLIALAFLSSSVNPVLYACAGGGLLRSAGV 298
                                                       TM7

guinea pig 298:GFVAKLLEATGSEAFSTRRGGTLAQTVKGIPMAPEPGASGSLDGLKQSESD---      348
human      299:GFVAKLLEGTGSEASSTRRGGSLGQTARSGPAALEPGPSESLTASSPLKLNELN      352
mouse      299:GFVVKLLEGTGSEVSSTRRGGTLVQTPKDTPACPEPGPTDSFMTSSTIPESSK-      351
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Figure 1 Comparison of amino acid sequences of guinea-pig, human and mouse BLT

The amino acid sequence of guinea-pig BLT is shown with those of human [11] and murine BLT [12]. The boxed amino acids are residues that are identical in the three species. The approximate positions of the putative transmembrane domains (TM) are underlined.

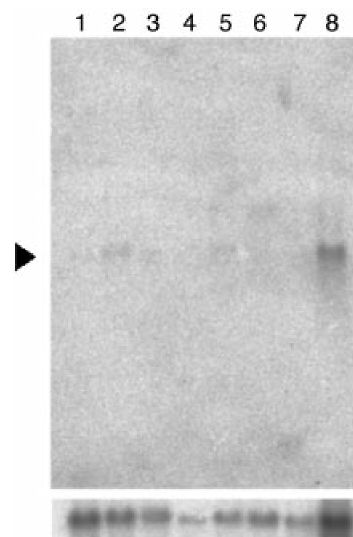

Figure 2 Northern blot analysis of BLT in various tissues of guinea-pig

Poly(A)$^+$ RNA species (6 μg) were applied as follows: lane 1, brain; lane 2, lung; lane 3, liver; lane 4, kidney; lane 5, spleen; lane 6, small intestine; lane 7, colon; lane 8, leucocytes. The membrane was hybridized with [^{32}P]dCTP-labelled ORF of guinea-pig BLT (upper panel) and glyceraldehyde-3-phosphate dehydrogenase (lower panel). The arrowhead indicates the guinea-pig BLT transcript.

The tissue distribution of guinea-pig BLT was investigated by Northern blot analysis of various guinea-pig tissues (Figure 2). The length of the positive bands was approx. 1.5 kb. The transcript was expressed at the highest level in leucocytes; very faint bands were also observed in lung and spleen. These results are consistent with previous reports showing specific binding for LTB_4 in various tissues of guinea-pig [17,18].

To examine the pharmacological profile of guinea-pig BLT, we transiently expressed the cDNA in HEK-293 and Cos-7 cells. As shown in Figure 3, the membrane fractions of HEK-293 cells

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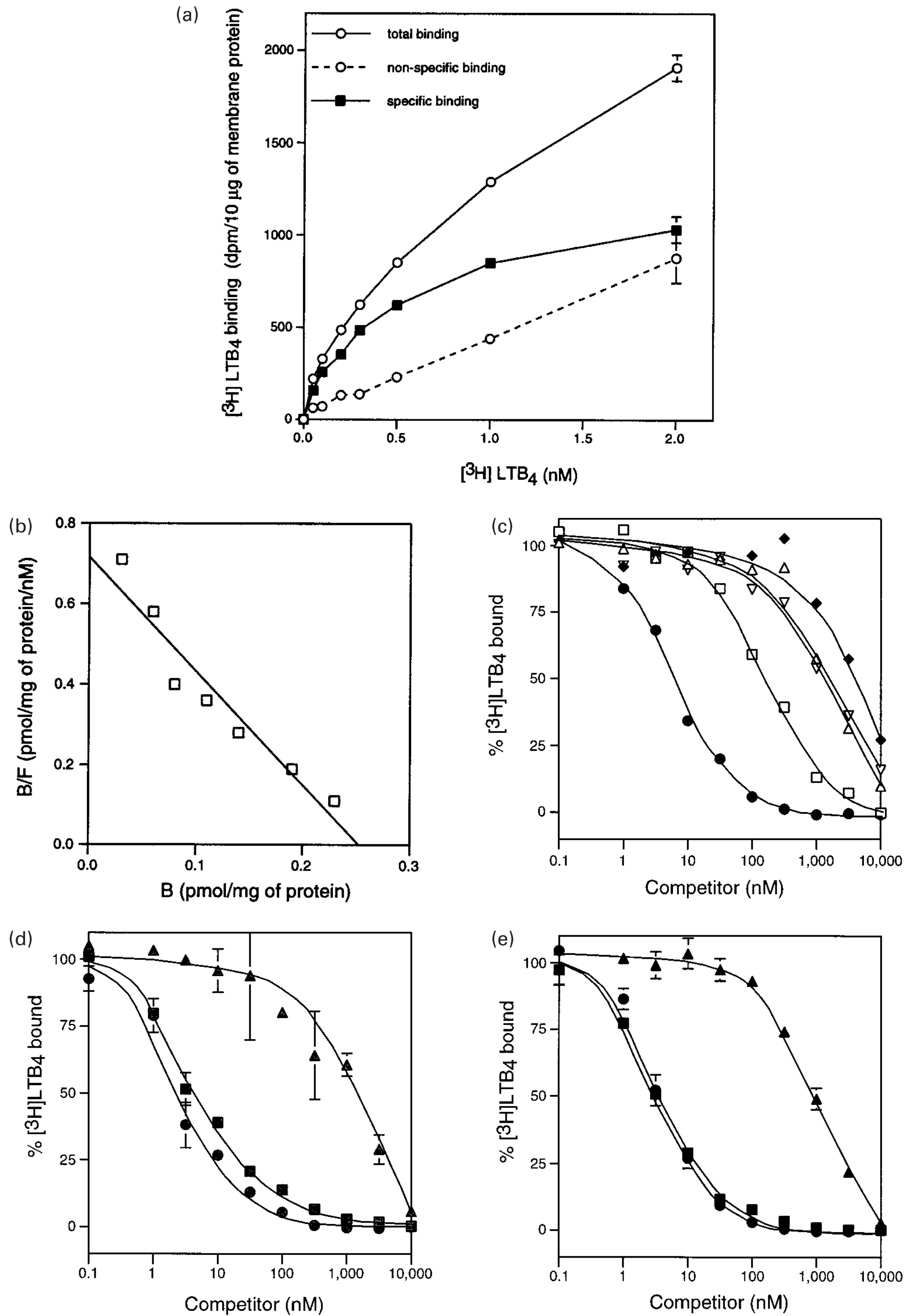

Figure 3 [^{3}H]LTB$_4$ binding to the membrane fractions of HEK293 cells transfected with pGPBR

(**a**, **b**) Binding isotherms (**a**) and Scatchard analysis (**b**) of guinea-pig BLT are shown. [$n = 4$, means ± S.D. in (**a**)] (**c**, **d**) Competition by various eicosanoids for 0.25 nM [^{3}H]LTB$_4$ binding to the membrane fraction of HEK293 cells transfected with pGPBR. Symbols in (**c**): △, 6-*trans*-LTB$_4$; ◆, 6-*trans*-12-epi-LTB$_4$; □, 12-oxo-LTB$_4$; ▽, 20-carboxy-LTB$_4$; ●, LTB$_4$; results are means ± S.D. ($n = 3$). Symbols in (**d**): ■, 20-hydroxy-LTB$_4$; ▲, (12*R*)-HETE; ●, LTB$_4$; results are means ± S.D. ($n = 4$). Because of the complexity, some error bars are not shown in (**c**). (**e**) Competition by 20-hydroxy-LTB$_4$ (■), LTB$_4$ (●) and (12*R*)-HETE (▲) for 0.25 nM [^{3}H]LTB$_4$ binding to the membrane fraction of guinea-pig leucocytes ($n = 4$, mean ± S.D.) The IC$_{50}$ values of the following eicosanoids were more than 10 μM: (5*R*)-HETE, (5*S*)-HETE, (12*S*)-HETE, (15*R*)-HETE, (15*S*)-HETE, 5-oxo-ETE, LTC$_4$, LTD$_4$, LTE$_4$, prostaglandin D$_2$, prostaglandin E$_2$, prostaglandin F$_2\alpha$ and platelet-activating factor.

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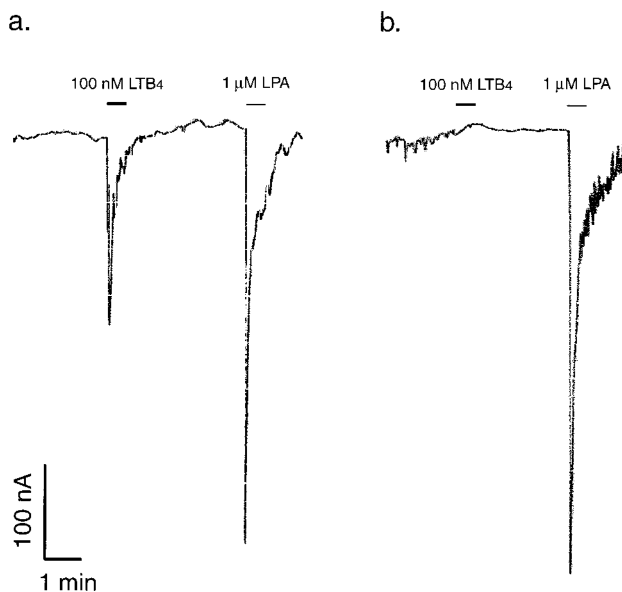

Figure 4 Typical Cl^- current responses of *Xenopus laevis* oocytes injected with cRNA of guinea-pig BLT

Cl^- currents were recorded from individual oocytes injected with 25 ng of guinea-pig BLT cRNA (**a**) or water (**b**). The solid bars above each trace represent the application of either 100 nM LTB_4 or 1 μM LPA. Both ligands showed maximal responses at these concentrations.

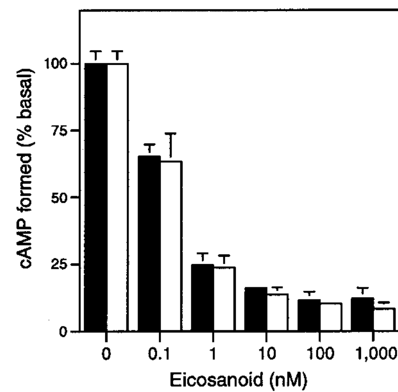

Figure 5 Inhibition of forskolin-induced cAMP formation in CHO-GPF10 by LTB_4 and 20-hydroxy-LTB_4

The inhibition of forskolin (50 μM)-induced cAMP formation was by LTB_4 (filled columns) or 20-hydroxy-LTB_4 (open columns) is shown ($n = 3$, means $\pm$ S.D.). The forskolin-induced cAMP levels in the absence of eicosanoids were 447.8 $\pm$ 37.8 pmol/10^6 cells.

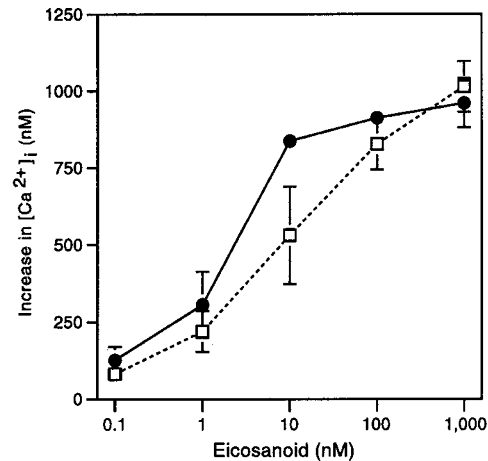

Figure 6 Increase in intracellular $[Ca^{2+}]$ in CHO-GPF10 cells by LTB_4 and 20-hydroxy-LTB_4

Increases in intracellular $[Ca^{2+}]$ induced by LTB_4 (●) and 20-hydroxy-LTB_4 (□) are shown ($n = 3$, means $\pm$ S.D.).

prepared 72 h after transfection displayed a dose-dependent and saturable binding for $[^3H]LTB_4$. The average K_d and B_{max} values from three independent experiments were 0.27 ± 0.079 nM and 425 ± 360 fmol/mg of protein respectively (means $\pm$ S.D., $n = 4$). The transfected Cos-7 cell membranes also showed similar binding properties of LTB_4, with a K_d of 0.17 nM and a B_{max} of 556 fmol/mg of protein (results not shown). There was no significant specific $[^3H]LTB_4$ binding in the membrane prepared from HEK-293 or Cos-7 cells transfected with the vector DNA (results not shown). The inhibition of $[^3H]LTB_4$ binding by various eicosanoids in the membrane fraction of HEK-293 cells transfected with guinea-pig BLT is shown in Figures 3(c) and 3(d). Both LTB_4 and 20-hydroxy-LTB_4 displayed the most efficient inhibition of $[^3H]LTB_4$ binding, followed by 12-oxo-LTB_4, 20-carboxy-LTB_4, 6-*trans*-LTB_4, 6-*trans*-12-epi-LTB_4 and (12*R*)-hydroxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid [(12*R*)-HETE]. Competition by 20-hydroxy-LTB_4 or (12*R*)-HETE for $[^3H]LTB_4$ binding to the membrane fraction of HEK-293 cells expressing guinea-pig BLT (Figure 3d) was similar to that of the guinea-pig leucocytes (Figure 3e). None of (5*R*)-HETE, (5*S*)-HETE, (12*S*)-HETE, (15*R*)-HETE, (15*S*)-HETE, 5-oxo-ETE, LTC_4, LTD_4, LTE_4, prostaglandin D_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$ or platelet-activating factor at 10 μM competed significantly for $[^3H]LTB_4$ binding to the transfected membrane fraction (results not shown). To exclude the possible conversion of LTB_4 to 20-hydroxy-LTB_4 or 12-oxo-LTB_4 during the binding assay, we analysed the lipid extracts by RP-HPLC (results not shown) and confirmed that LTB_4 was intact in the binding assay. These binding profiles of LTB_4 to guinea-pig BLT agree well with pharmacological data obtained by using various tissues of guinea-pig [17–19], confirming that mgpl-2 and mgpl-3 encodes guinea-pig BLT.

Ca^{2+}-dependent Cl^- current in *Xenopus laevis* oocytes is widely used for analysing receptor functions [30–32]. The hydrolysis of inositol phospholipids by phospholipase C forms Ins(1,4,5)P_3, resulting in the increase in intracellular $[Ca^{2+}]$, which in turn opens the Ca^{2+}-dependent Cl^- channel in oocytes. When the *Xenopus* oocytes were injected with cRNA of the guinea-pig BLT, they showed LTB_4-induced Cl^- currents (48–172 nA). A typical response is shown in Figure 4; the LTB_4-induced Cl^- currents were dependent on the amount of the BLT cRNA (results not shown). Although oocytes injected with distilled water showed no response to LTB_4, they showed significant responses to 1 μM lysophosphatidic acid (LPA) through the intrinsic LPA receptor(s) expressed in the oocytes [33–36], suggesting that the cloned cDNA codes for the functional receptor for LTB_4.

To examine the intracellular signalling by LTB_4 through guinea-pig BLT, we established CHO cells stably expressing guinea-pig BLT. As shown in Figure 5, both LTB_4 and 20-

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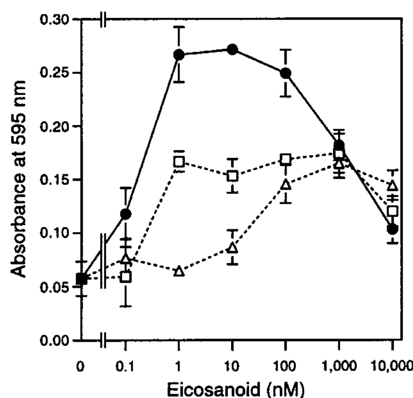

Figure 7 Chemotactic response of CHO-GPF10 cells induced by eicosanoids

CHO-GPF10 cells showed chemotactic activities in the Boyden chamber assay towards LTB_4 (●), 20-hydroxy-LTB_4 (□), and 20-carboxy-LTB_4 (△) ($n = 4$, means ± S.D.).

hydroxy-LTB_4 inhibited forskolin-induced cAMP formation in a dose-dependent manner. The IC_{50} values of LTB_4 and 20-hydroxy-LTB_4 were 0.56 and 0.46 nM respectively, suggesting that LTB_4 and 20-hydroxy-LTB_4 activate G_i-like G-protein to the same extent. We next measured Ca^{2+} mobilization by using CHO-GPF10 cells (Figure 6). LTB_4 increased intracellular [Ca^{2+}] in CHO-GPF10 cells. Although 20-hydroxy-LTB_4 was less effective than LTB_4, it increased intracellular [Ca^{2+}] in a dose-dependent manner. (12*R*)-HETE (1 μM) did not significantly increase intracellular [Ca^{2+}] (results not shown).

To compare the biological effects of LTB_4 and its metabolites, we performed a chemotaxis assay in CHO-GPF10 cells with the Boyden chamber method. As shown in Figure 7, LTB_4-induced chemotactic activity exhibited a bell-shaped dose dependence, the activity being highest with 1 and 10 nM LTB_4. Although 20-hydroxy-LTB_4 exhibited chemotaxis, the activity was approximately half that of LTB4. 20-Carboxy-LTB4 showed weak chemotactic activity: 1 μM concentration was required for a maximum response.

DISCUSSION

We cloned a guinea-pig BLT cDNA and characterized the receptor. The cloned receptor is a G-protein-coupled receptor with seven putative transmembrane domains, and is 73 % and 70 % identical with human and murine BLT respectively at the amino acid levels (Figure 1). The identity was high in the second and seventh transmembrane domains between human, murine and guinea-pig BLTs. Homologous and heterologous desensitization of LTB_4 responses were reported in human neutrophils and human monocytic leukaemia U-937 cells [37,38]. Guinea-pig BLT contains several potential phosphorylation sites that might be involved in the desensitization [29] (Figure 1). Although amino acid residues in the third intracellular loop of human BLT are identical with those of murine BLT, they are 70 % identical with those of guinea-pig BLT. Numerous experiments have shown that receptor/G-protein coupling occurs through the second and third intracellular loops or through the C-terminal cytoplasmic tail of the receptors [39]. The conserved amino acids in these cytoplasmic domains might be important for the G-protein coupling of BLT.

Northern blot analysis of guinea-pig tissues revealed that the pattern of BLT expression differs from that of human tissues. In our previous report, two cDNA species (1.7 and 3.0 kb in length) with distinct 5′-untranslated regions were isolated from a leukaemia cell line, HL-60 [11]. Northern blotting showed that the two corresponding bands of mRNA were observed in HL-60 and U-937 cells and that the highest expression was observed in leucocytes, followed by spleen and thymus. In contrast, the guinea-pig BLT transcripts were detected as a single band 1.5 kb in length (Figure 2). Very faintly hybridized bands of the same length were observed in lung and spleen in guinea pig as well as in leucocytes (Figure 2). The Northern blot analysis correlates well to the previous reports showing that specific binding activity for LTB_4 was observed in leucocytes, lung and spleen of guinea pig [16–20]. Studies on the transcriptional regulation of human BLT are continuing in our laboratory; it might well be that BLT expression in human is regulated differently from that in guinea-pig.

Previous studies showed that the K_d of BLT of various guinea-pig tissues were in the nanomolar range [17–19]. LTB_4 binds to membrane fractions of HEK-293 cells transiently expressed with guinea-pig BLT with a K_d value of 0.27 nM (Figure 3). The BLT-transfected Cos-7 cell membrane fractions also showed similar results. In addition, *Xenopus laevis* oocytes that were injected with guinea-pig BLT cRNA showed LTB_4-induced Cl^- currents (Figure 5). All these results suggest that the cloned cDNA encodes a functional receptor for LTB_4.

LTB_4 is metabolized to 20-hydroxy-LTB_4 by cytochrome P450 (ω-oxidation) and is further converted to 20-carboxy-LTB_4 in human granulocytes [23,40]. Alternatively, it is metabolized to 12-oxo-LTB_4 by LTB_4 12-hydroxydehydrogenase [41]. These metabolites have been considered as inactive forms of LTB_4. Unexpectedly, we detected no significant difference between LTB_4 and 20-hydroxy-LTB_4 in displacement of [^{3}H]LTB_4 binding to the membrane fractions of HEK-293 cells transfected with guinea-pig BLT cDNA (Figure 3d). Similar results were obtained for the membrane fraction of guinea-pig leucocytes (Figure 3e). LTB_4 was not metabolized to 20-hydroxy-LTB_4 or to 12-oxo-LTB_4 during the binding assay, as determined by RP-HPLC analyses (results not shown). The rank order of the compounds for inhibition of [^{3}H]LTB_4 binding was LTB_4, 20-hydroxy-LTB_4 12-oxo-LTB_4 20-carboxy-LTB_4, 6-*trans*-LTB_4, 6-*trans*-12-epi-LTB_4 and (12*R*)-HETE.

Then we examined intracellular events evoked by LTB_4 and 20-hydroxy-LTB_4 with CHO-GPF10 cells. The forskolin-induced accumulation of cAMP was inhibited by 20-hydroxy-LTB_4 as well as by LTB_4 (Figure 5). These results suggest that both LTB_4 and 20-hydroxy-LTB_4 bind to guinea-pig BLT and activate G_i protein(s) with the same efficiency. However, Ca^{2+} mobilization elicited by 20-hydroxy-LTB_4 was less potent than that by LTB_4 (Figure 6). A more prominent difference was seen between LTB_4 and 20-hydroxy-LTB_4 regarding chemotactic activity (Figure 7). We reported previously that the Ca^{2+} mobilization was inhibited only partly by treatment with *Bordetella* pertussis toxin [11]; it has also reported that $G\alpha_{16}$ is also involved in the LTB_4-induced activation of phospholipase $C\beta$ [42]. Chemotaxis might require activation of the $\beta\gamma$ subunit of G_i protein(s) [43,44], because it is inhibited by treatment with pertussis toxin [11]. It is also reported that the Rho family proteins Rho, Rac and Cdc42 are important for cell migration [45–47] and that G_{13} mediates the activation of Rho [48]. The precise mechanism underlying the different effects between LTB_4 and 20-hydroxy-LTB_4 is not clear. It is speculated that these compounds activate several signalling molecules other than G_i with different efficiencies even though both bind to the receptor with similar affinities. The present observation poses a new question regarding the relationship between the receptor binding and effector activation through different types of G-protein.

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In conclusion, we have cloned and characterized guinea-pig BLT. The comparison of the amino acid sequences of BLT from three species makes feasible the rational design of BLT antagonists, which are candidates for anti-inflammatory drugs. Our study also paves the way for determining the interaction between LTB_4 and BLT by site-directed mutagenesis and for the isolation of novel receptors with structural similarities to BLT.

We thank M. Nakamura (University of Tokyo, Tokyo, Japan) for providing the guinea-pig leucocyte cDNA library; Ono Pharmaceutical Co. (Osaka, Japan) for supplying LTB_4; and Dr. S. Tabuchi, Dr. K. Kume, Dr. I. Ishii, Dr. S. Ishii, Dr. M. Taniguchi and Dr. T. Igarashi for discussion. This work was supported in part by grants-in-aid from Ministry of Education, Science, Culture and Sports, CREST of Japan Science and Technology and the Human Science Foundation, and grants from Yamanouchi Foundation for Metabolic Disorders and the ONO Medical Research Foundation.

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Received 11 March 1999/24 May 1999; accepted 11 June 1999

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