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Guinea pig cysteinyl leukotriene receptor 2 (gpCysLT2) mediates cell proliferation and intracellular calcium mobilization by LTC4 and LTD4

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We cloned and pharmacologically characterized the guinea pig cysteinyl leukotriene (CysLT) 2 receptor (gpCysLT2). gpCysLT2 consists of 317 amino acids with 75.3%, 75.2%, 73.3% identity to those of humans, mice and rats, respectively. The gpCysLT2 gene is highly expressed in the lung, moderately in eosinophils, skin, spleen, stomach, colon, and modestly in the small intestine. CysLTs accelerated the proliferation of gpCysLT2-expressing HEK293. Leukotriene C4 (LTC4) and Leukotriene D4 (LTD4) enhanced the cell proliferation higher than Bay-u9773, a CysLT2 selective partial agonist and a non-selective antagonist for CysLT receptors. Bay-u9773 did not antagonize the cell proliferation by LTC4 and LTD4. Despite the equipotency of the mitogenic effect among these chemicals, calcium mobilization (CM) levels were variable (LTC4 > LTD4 >> Bay-u9773), and Bay-u9773 antagonized the CM by LTC4. Moreover, the Gi/o inhibitor pertussis toxin perfectly inhibited agonist-induced cell proliferation. These results reveal that cell proliferation via CysLT2 signaling was mediated by Gi/o signaling but independent of calcium mobilization. [BMB reports 2008; 41(2): 139-145]

INTRODUCTION

Cysteinyl leukotrienes (CysLTs) are pro-inflammatory mediators synthesized from membrane-bound arachidonic acid through the 5-lipoxygenase (5-LO) pathway (1). 5-LO, which translocates from the cytosol to the nuclear membrane, produces intermediate leukotriene A_4 (LTA4), which is immediately converted into either leukotriene B_4 (LTB4) or CysLTs (2). Recently, another pathway of biosynthesis of leukotrienes has been revealed. A cell possessing 5-LO including neutrophil synthesizes the unstable metabolic intermediate LTA4, which can be discharged and converted into CysLTs by vicinal acceptor cells with LTA4-hydrolase or LTC4-synthase. This process has been termed transcellular biosynthesis. This indicates that inflammatory cells form a favorable milieu for the production and control of leukotriene biosynthesis (3-5), and may affect systemic organ function (4, 6). CysLTs mediate their effects by binding to specific cell surface receptors designated CysLT1 and CysLT2, belonging to the seven-transmembrane, G protein-coupled receptor family. CysLT1 and CysLT2 have been shown to exert distinct functions (7). The presence of an intracellular cysteinyl leukotriene receptor has been proposed (8).

A variety of clinical and experimental studies have demonstrated the function of CysLT1 using specific receptor antagonists. Through CysLT1, CysLTs mimic the features of asthma, including mucus secretion, inflammatory cell recruitment, edema, neuronal dysfunction, and bronchoconstriction (9). In CysLT2, there is increasing evidence of an important role in chronic inflammation. Furthermore, genetic analysis revealed that human CysLT2 locus (13q14) is associated with atopic asthma (10). Airway structural changes in asthma with persistent inflammation and airway remodeling presents airway wall thickening, subepithelial fibrosis, and hyperplasia of the mucus glands, myofibroblasts, smooth muscle, and vasculature (11). CysLT2 is involved in airway smooth muscle cell hyperplasia and subepithelial fibrosis (12). CysLT2 mediates proliferative response in human airway smooth muscle cells (HASM) and human mesangial cells (13). It was reported that proliferation of CHOK1 cells was induced through PDGFRβ by LTD4. Moreover, Yoshisue *et al*. demonstrated that LTD4 induced cell proliferation via neither CysLT1 nor CysLT2 (14). Because of limitation of cell lines and organs of exclusive expression of CysLT2, Bay-u9773, nonselective cysteinyl leukotriene receptors antagonist, was frequently used in past studies for CysLT2 function. However, recent studies using target disruption of mouse CysLT2 have revealed the involvement of CysLT2 in chronic pulmonary inflammation with fibrosis (15).

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Since CysLT2 is expressed in endothelium, eosinophils and mast cells and stimulates endothelial cell adherence, myofibroblast proliferation, and chemokine production by mast cells, combinatorial antagonists of both CysLT1 and CysLT2 receptor are required for the inhibition of the proinflammatory activities of CysLTs (16).

Guinea pigs have been used in the experimental research on leukotrienes including bronchoconstriction (17). The presence of CysLT2 in guinea pigs (18) has long been suspected even before cloning of human CysLT2. However, the guinea pig CysLT2 cDNA sequence has not been revealed. Therefore, we report the cDNA encoding of guinea pig CysLT2 (gpCysLT2) (**AY236969**). Here we also describe the *in vitro* function of CysLT2.

RESULT AND DISCUSSION

Cloning of gpCysLT2 cDNA

We performed PCR based cloning using a cDNA library derived from guinea pig eosinophils. The obtained PCR product contained sequences with high homology to ORF of CysLT2 in other species. Then full length ORF was obtained by 5' and 3' RACE. The ORF of gpCysLT2 was 954 bp in length and encoded 317 amino acid sequences, which was shorter than human CysLT2 by 29 residues (Supplemental Fig. 1). Hydrophobicity analysis suggested that there were seven transmembrane-spanning regions (Fig. 1A). Conserved motifs in the rhodopsin-like G protein-coupled receptor family were identified. For example, two Cys residues in the first and second extracellular loops, Asp in transmembrane domain 2 (TM2), Trp in TM4, Tyr in TM5, and Pro in TM6, were all present in gpCysLT2 (Fig. 1B). Alignment analysis revealed that the deduced protein sequence shared 75.3%, 75.2% and 73.3% homology with that of its human, mouse and rat counterparts, respectively.

Tissue distribution of gpCysLT2 expression

Based on functional smooth muscle studies, it was reported that gpCysLT2 was expressed in the ileum (18) and trachea (19). We investigated the tissue distribution of guinea pig CysLT2 gene by quantitative real time PCR (Fig. 2). gpCysLT2 was highly expressed in the lung. It is moderately expressed in eo-

A

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MEPNNSSSRNCMIQESFKKEFYPVTYLVIFVWGALGNGLSIYVFLQTYKKSTSANVFMLNLAMSDLLFISTLP
FRAHYYLNNSNWIFGDVPCRIMSYSLYVNMYTSIYFLTVLSVVRFLATVHPFRLLHVTSFRSAWILCGIIWIF
TMASAAVLLMHGSEPKNSITTCLELDIRKVGKLKVMNHIALVVGFLLPFFTLSICYLLVIRVLLKVEIPESTL
RASHRKALITIIIALITFLLCFLPYHTLRTLHLITWNKDSCGNGLHKAVVITLALAAANSCVNPFLYYFAGEN
FKDKLKAVFIKDHPQKAKCSFPICL
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B

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gpCysLT2    1:----------------MEPNNSSS----RNCMIQESFKKEFYPVTYLVIFVWGALGNGLS 40
hsCysLT2    1:MERKFMSLQPSISVSEMEPNGTFSNNNSRNCTIEN-FKREFFPIVYLIIFFWGVLGNGLS 59
mCysLT2     1:----------------MEVTGTPSSYSNRNCTIEN-FKKEFYPIIYLIIFFWGALGNGFS 43
rCysLT2     1:----------------MGVTGTPSYYSDKNCTIEN-FKRDFYPIIYLIIFVWGALGNGFS 43

gpCysLT2   41:IYVFLQTYKKSTSANVFMLNLAMSDLLFISTLPFRAHYYLNNSNWIFGDVPCRIMSYSLY 100
hsCysLT2   60:IYVFLQPYKKSTSVNVFMLNLAISDLLFISTLPFRADYYLRGSNWIFGDLACRIMSYSLY 119
mCysLT2    44:IYVFLQTCKKSTSVNVFMLNLATSDFLFISTLPFRADYYFRGSNWIFGDLACRVMSYSLY 103
rCysLT2    44:IYVFLQTYKKSTSVNVFMLNLAISDFLFISTLPFRADYNFRGSDWIFGDWACRIMSYSLY 103

gpCysLT2  101:VNMYTSIYFLTVLSVVRFLATVHPFRLLHVTSFRSAWILCGIIWIFTMASAAVLLMHGSE 160
hsCysLT2  120:VNMYSSIYFLTVLSVVRFLAMVHPFRLLHVTSIRSAWILCGIIWILIMASSIMLLDSGSE 179
mCysLT2   104:VNMYTSIYFLTVLSVVRFQATVHPFRMFHVTSVRSAWILCGIIWVFIMASSALLIVNGQE 163
rCysLT2   104:VNMYTSIYFLTVLSIVRFLATAHPFQMIHITSVRSAWILCGIIWVFIMASSGLLLKHGQE 163

gpCysLT2  161:PKNSITTCLELDIRKVGKLKVMNHIALVVGFLLPFFTLSICYLLVIRVLLKVEIPESTLR 220
hsCysLT2  180:QNGSVTSCLELNLYKIAKLQTMNYIALVVGCLLPFFTLSICYLLIIRVLLKVEVPESGLR 239
mCysLT2   164:EKDNIISCLELSPQKFKSLLIMNHIAVAVGFLLPFLTLTVCYLLIIRILLKAEIPESGPR 223
rCysLT2   164:KKNNTTLCFELNLQKFKNLVIINMIALGVGFLLPFFILTICYLLIIRVLLKVEIPESGPR 223

gpCysLT2  221:ASHRKALITIIIALITFLLCFLPYHTLRTLHLITWNKDSCGNGLHKAVVITLALAAANSC 280
hsCysLT2  240:VSHRKALTTIIITLLIFFLCFLPYHTLRTVHLTTWKVGLCKDRLHKALVITLALAAANAC 299
mCysLT2   224:AAHRKALTTIVIAMITFLLCFLPYHALRTLHLVTWDKDSCGDVLHKATVITLTMAAANSC 283
rCysLT2   224:DAQRKALTTIVIAMIIFLLCFLPYHALRTIHLVTWDADSCMDELHKATVITLTLAAANSC 283

gpCysLT2  281:VNPFLYYFAGENFKDKLKAVFIKDHPQKAK--CSFPICL-------- 317
hsCysLT2  300:FNPLLYYFAGENFKDRLKSALRKGHPQKAKTKCVFPVSVWLRKETRV 346
mCysLT2   284:FNPFLYYFAGENFKARLRAIFSKVHL---------------------- 309
rCysLT2   284:FNPFLYYFAGENFKARLRAIFSKDHL---------------------- 309
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Fig. 1. Characterization of the gpCysLT2 sequence. (A) Amino acid sequences of gpCysLT2 (AY236969). The putative transmembrane domain is underlined. The prediction of 7 transmembrane domains was performed using TMPred (http://www.ch.embnet.org/software/TMPRED_form. html). (B) Sequence alignment of the amino acid sequences of human, mouse, rat CysLT2. Light letters on a dark background identify identical amino acids. Asterisks indicate the conserved residues in the rhodopsin family G-protein-coupled receptor.

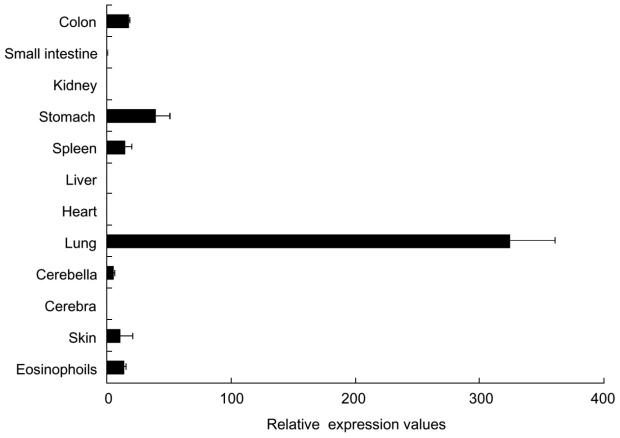

Fig. 2. Tissue distribution of guinea pig CysLT2 gene. cDNA was generated from three guinea pigs. Tissue distribution was measured by quantitative real time PCR. Relative expression values (gpCysLT2/GAPDH) and experimental variance (SE) are shown (n = 3).

sinophils, skin, cerebella, spleen, stomach, colon, and modestly expressed in the small intestine. Our results are consistent with previous reports for the other animals. However, expression of the CysLT2 gene varies by strain and species. Although CysLT2 was reported to be expressed in the human heart (20) and mouse (21), the C57BL/6 mouse lacked CysLT2 in the heart (22).

Induction of cellular proliferation by CysLTs

We assessed the induction of cell proliferation through CysLT2 in HEK293 stably expressing gpCysLT2 (HEKgpCysLT2). Confocal microscopy showed that gpCysLT2 were expressed largely on the cell membrane under the cytomegalovirus (CMV) promoter-based expression vector (Supplemental Fig. 2). The stimulation of LTC4 and LTD4 for 48 h with 5% FBS condition induced a 1.5-fold increase in the cell number of HEKgpCysLT2. LTE4 and LTB4 showed no response (Fig. 3A). Cell proliferation response through CysLT2 with LTD4 has been reported in CHOK1 cells with exclusive CysLT2 expression (13). Our experiments also demonstrated the proliferative activity of LTD4 through CysLT2. Here, we found the proliferative function of LTC4 to be at the same level as that of LTD4. No leukotrienes increased in cell number in vector control cells. Since these proliferative responses were not observed under serum free condition (data not shown), some serum factors seemed to be required to promote cellular proliferation in HEKgpCysLT2 as well as the other cells reported previously (12). Then, we examined the effects of cysteinyl leukotriene receptor antagonists (Fig. 3B). MK571, CysLT1 antagonist, did not suppress cell proliferation. Interestingly, pretreatment of Bay-u9773, a selective agonist for CysLT2 (23) and a nonselective antagonist for CysLT1 and CysLT2, did not inhibit cell proliferation, but promoted it (Fig. 3B, C). These proliferative responses were abrogated with 100 ng/ml PTX (Fig. 3D). These results indicated that the proliferative responses were mediated by exogenously transfected gpCysLT2, and that LTD4, LTC4 and Bay-u9773 would promote cellular proliferation through PTX sensitive G-protein (Gi/o-protein).

Intracellular calcium elevation through gpCysLT2

LTC4 and LTD4 evoked a dose-dependent increase in intracellular calcium in HEKgpCysLT2. More than a hundred-fold concentration of LTC4 was needed for the same level as LTD4-inuduced calcium mobilization (Fig. 4A). Bay-u9773 itself evoked a slight increase in intracellular calcium (Fig. 4B). Despite the equipotency of the mitogenic effect among these chemicals (Fig. 3), calcium mobilization levels were variable (LTC4 > LTD4 >> Bay-u9773). This indicated that cell proliferation by agonist-gpCysLT2 signaling was independent of calcium mobilization. Bay-u9773 was reported to inhibit intracellular calcium mobilization by the CysLTs (23). In this study, the intracellular calcium response to LTC4 was not inhibited by 1 μM of MK-571, CysLT1 antagonist, but was inhibited by 1 μM of Bay-u9773 (Fig. 4C). Previous reports showed functional antagonism of Bay-u9773 to the contraction activities of CysLT2 in organ bath assay of guinea pig (18, 19). We showed that Bay-u9773 behaved as an antagonist and a partial agonist for gpCysLT2 in intracellular calcium response as well as other species (23) and that Bay-u9773 induced cellular proliferation by itself and with the following stimulation of LTC4. Bay-u9773 bound to gpCysLT2 with a high affinity (24). It is likely that Bay-u9773 provoke signal transduction for proliferation through CysLT2 in common with LTC4 independent of calcium signaling.

In conclusion, we first cloned gpCysLT2 and found that it was an orthologous gene for the known CysLT2 receptors in the other species, and that its homology to the human counterpart was closer than that in the other rodents. This evidence indicated that the guinea pig model, as well as the human being, was very sensitive to chemical mediators compared with other rodent model organisms e.g. mouse and rat. Ressmeyer *et al.* showed similar response between human and guinea pig lung slices to LTD4 (17). It may be reflected on the frequent use of the guinea pig as the asthma model and in leukotrienes research (25). Moreover, we revealed that LTC4 and LTD4 had the same potency for cellular proliferation through CysLT2, despite LTC4 having much higher potency for increasing intracellular calcium than LTD4. CysLT2 may be involved in airway remodeling in chronic airway diseases. In asthmatic patients, CysLT2 signaling could promote proliferation of some kinds of cells, such as airway smooth muscle and fibroblast through Gi/0 signaling independent of calcium signaling. The development of functionally specific CysLT2 antagonists, which specifically inhibits Gi/0 signaling, might open the way to an alternative therapeutic procedure for chronic airway diseases.

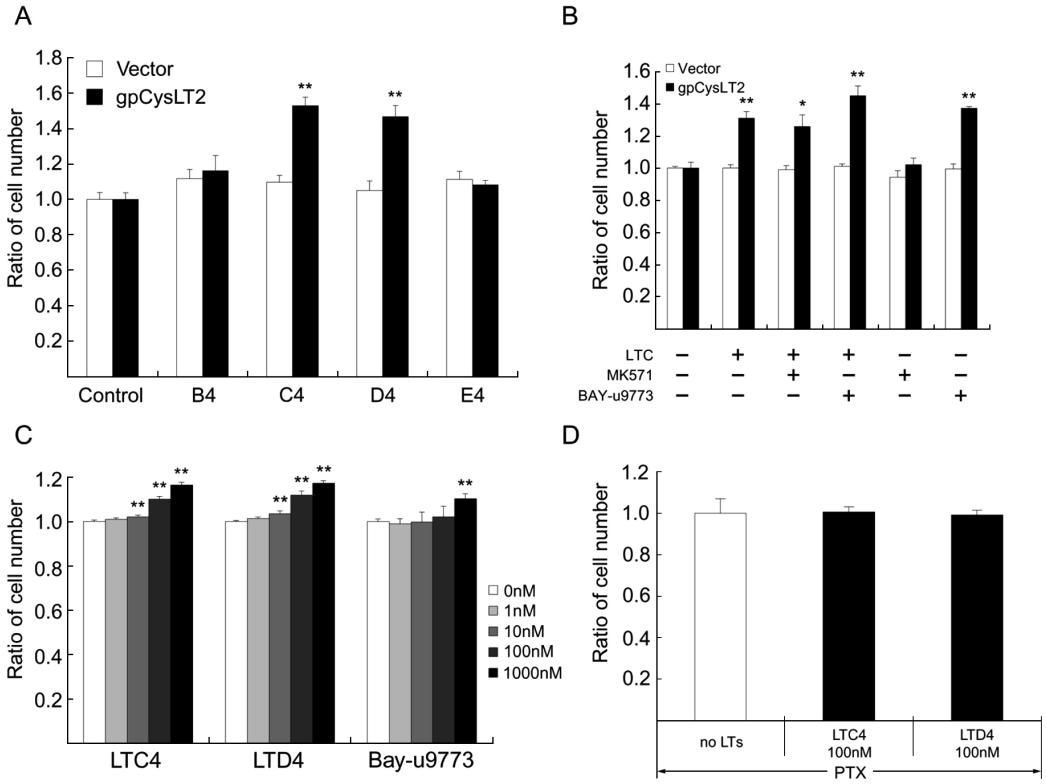

Fig. 3. Induction of cellular proliferation by CysLTs in HEKgpCysLT2. Cells were cultured for 24 h and then incubated with/without LTC4 and LTD4 (each 10^{-7} M) in the 5% serum and serum free medium. The cell number was measured after 48 h (A), 48 h (B) and 24 h (C) incubation with CysLTs. (A) The changes of cell number by CysLTs were expressed as a ratio in the presence of 5% serum. (control n = 16, others n = 4, means ± S.E.). LTC4 and LTD4 increased the ratio of cell number at the same level, but LTB4 and LTE4 did not. Statistically significant differences compared with untreated control in each cell are indicated, *P < 0.05, **P < 0.01, Scheffe's F-test. (B) The changes of cell number by LTC4 and antagonists were expressed as a ratio compared to control in HEKgpCysLT2 (n = 3, means ± S.E.) and vector cells. 2 uM MK571 and 2 uM Bay-u9773 were added 15 min before the stimulation of LTC4. LTC4 increased the ratio of cell number and MK-571 did not significantly suppress LTC4-induced cellular proliferation. Bay-u9773 alone increased cell number in HEK293 cells stably expressing gpCysLT2. Interestingly, Bay-u9773 did not inhibit LTC4-induced cellular proliferation but induced partially additive increase. In vector cells no significant changes were observed. Statistically significant differences compared with untreated control in each cell are indicated, *P < 0.05, **P < 0.01, Scheffe's F-test. (C) The changes of cell number by LTC4, LTD4 and Bay-u9773 were expressed as a ratio compared to control in HEKgpCysLT2 (n = 3, means ± S.E.). LTC4 and LTD4 increased the ratio of cell number at the same content in a dose dependent manner. Bay-u9773, reported to be a partial agonist for CysLT2, also increased cell number. MK-571 did not significantly suppress LTC4-induced proliferation. Statistically significant differences compared with untreated control in each cell are indicated, *P < 0.05, **P < 0.01, Scheffe's F-test. (D) The inhibition of cellular proliferation by PTX in HEKgpCysLT2 (n = 6, means ± S.E.) and vector cells. The pre-incubation of PTX (100 ng/ml) completely diminished cellular proliferation by LTC4 and LTD4. Cells were incubated with PTX for 24 hr before the addition of leukotrienes.

MATERIALS AND METHODS

Reagents

LTB4, LTC_4, LTD_4 and LTE4 (Cayman chemical), MK-571 and BAY-u9773 were purchased from BIOMOL Research (Plymouth Meeting). Pertussis toxin (PTX) was purchased from LIST BIOLOGICAL LABORATORIES, INC. (Campbell.). For preparing stock solutions, PTX was resolved in water and the others were resolved in dimethyl sulfoxide.

Purification of guinea pig peritoneal eosinophils

Eosinophil purification was carried out according to a previously described method (26) with slight modification. Briefly, male Hartley guinea pigs (700 to 800 g) were treated with undiluted horse serum (1 mL intraperitoneal injection) once a week for 8 weeks. The guinea pigs were anesthetized, and the cells were harvested by peritoneal lavage with normal saline 2 days after the last injection. The cells obtained were layered onto a discontinuous Percoll-Hanks' balanced salt solution (HBSS) gradient followed by centrifugation (1,500 g for 25 minutes at 20°C). The cells were then washed twice in

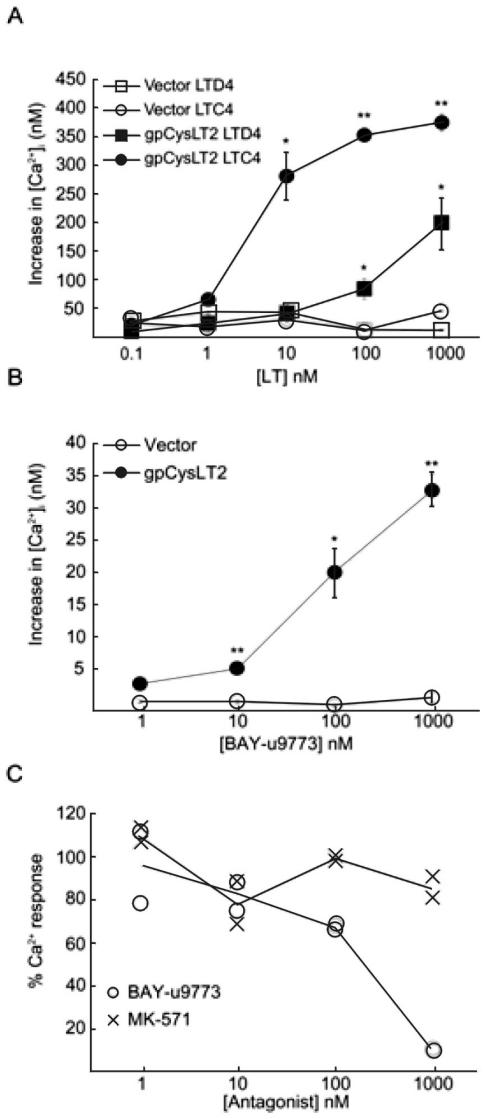

Fig. 4. Ca^{2+} mobilization in HEK293 cells stably expressing gpCysLT2 (HEKgpCysLT2). HEKgpCysLT2 were loaded with Fluo3 -AM and challenged with LTC4 or LTD4. (A) Intracellular calcium increases challenged with LTC4 or LTD4 in HEKgpCysLT2 and vector control cells are shown (n = 4, means ± S.E.). Statistically significant differences between the control and HEKgpCysLT2 are indicated, *P < 0.05, **P < 0.01, unpaired *t*-test. (B) Intracellular calcium increases challenged with Bay-u9773 in HEKgpCysLT2 and vector control cells are shown (n = 4, means ± S.E.). Bay-u9773 induced lower increase of intracellular calcium than LTC4. Statistically significant differences between the control and HEKgpCysLT2 are indicated, *P < 0.05, **P < 0.01, unpaired *t*-test. (C) Effect of CysLT antagonists was examined. The increase in intracellular calcium after 10 nM LTC4 stimulation is shown as a percentage to that of the cells without an antagonist (n = 2, each replicate was shown). BAY u9773 inhibited the response to LTC4, whereas MK-571 did not affect the response to the LTC4 stimulation. The experiments were performed in the presence of 5 mM serine and 10 mM borate.

HEPES buffer (HBSS, 10 mM HEPES pH 7.4, 0.1%w/v BSA). Eosinophils (> 90% pure, > 99% viable) were collected from the 1.090/1.095 and 1.095/1.112 g/mL density interfaces.

Cloning of gpCysLT2

Total RNA was extracted from guinea pig eosinophil and reverse-transcribed using Superscript II (Invitrogen) and oligo d(T)18 primer. Partial sequences of the gpCysLT2 ORF were obtained by PCR using degenerate primers derived from well-conserved regions of published CysLT2 receptors, sense primer (5'-TTCCTTRTATGTCAACATGTA-3') and antisense primer (5'-CCCCAGCAAARTAATAGA-3) (11, 22, 27), (AB052661). To obtain ORF of gpCysLT2, 5' and 3' RACE were carried out using a GeneRacer Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The 5' ends of ORF were amplified by PCR using Advantage2 polymerase (Clontech) with the GeneRacer 5' primer, GeneRacer 5' nested primer, the following gene specific primers (GSP), antisense primer (5'-ccacctttaacaggacgcgaatgac-3') and antisense nest primer (5'-cagcttacccaccttgcggatgtct -3'). The 3' end of ORF was performed with the GeneRacer 3' primer and the following GSP, sense primer (5'- ggcttcctgctgcccttcttcac -3'). The PCR products were subcloned into pCR®4-TOPO® vector (Invitrogen) for sequencing. These clones were sequenced using an automated DNA sequencer 377 (Applied Biosystems, Foster City, CA).

Stable expression of gpCysLT2

The full-length gpCysLT2 ORF were amplified by PCR with the following primer pairs, sense primer (5'- tcgaattcATGGAACC-AAACAATAGCAG-3') and antisense primer (5'-gcggatccCA-GACAAATAGGAAAACTAC-3'). PCR products were digested with restriction enzymes (BamHI and XhoI), and subcloned into an expression vector, pIRESneo2 (Clontech) between the BamHI and the XhoI site. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. To obtain HEK-293 cells stably expressing gpCysLT2, the cells were transfected with pIRES neo2-gpCysLT2 using TfxTM-20 (Promega) and selected with 500 μg/ml G418 (Clontech). HEK-293 cells transfected with the no-insert vector were also kept in a medium with G418 and used as a vector control.

Quantification of tissue distribution of gpCysLT2 by quantitative real time PCR

Tissue was collected from 4 anesthetized male Hartley guinea pigs (8 weeks). Gathered tissue was cut into small pieces and immediately submerged in RNAlater RNA stabilization Reagent (QIAGEN). Total RNA purification and DNase treatment were performed by RNeasy Midi Kit and RNase-Free DNase Set (QIAGEN). cDNA was generated from 3 ug of total RNA using Superscript II (Invitrogen) and oligo d(T)18 primer, then diluted up to 50 μl with TE. Quantitative real time PCR. Target gene amplification by PCR was performed with follow-

ing primers, for gpCysLT2 (GenBank accession Number: **AY236969**), forward primer (5'- ctgggaaacggcctgtcaat -3') and reverse primer (5'- ttctgaagctggtgacgtggag -3'), for GAPDH (GenBank accession Number: **AB060340**), forward primer (5'- tctgggaagctgtggcgtgat -3') and reverse primer (5'- ggaagaatggctgtcactgttg-3'). The final component of the real-time PCR solutions for 1 well (total 10 ul) is as follows, 1 × PCR buffer II, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.3 μM forward primer, 0.3 μM reverse primer, 0.5 × SYBR Green I (Molecular Probes), 1 μM 5- and 6-Carboxy X Rhodamine (Research Organics), 0.2 mg/ml BSA, 2% (v/v) Tween 20, 0.15 M trehalose, 0.01 unit AmpliTaq Gold (Applied Biosystems), 0.5 μl template cDNA solution. Real-time PCR was conducted by ABI PRISM 7700 Sequence Detector (Applied Biosystems). Thermal cycling and quantification were performed by Sequence Detection Systems 1.7. Thermal cycling, stage 1: 50°C 2 min, stage 2: 95°C 10 min, stage 3: 95°C 15 s, 55°C 30 s, 72°C 30 s. Stage 3 was repeated for 40 cycles. Each sample was analyzed in triplicate, and the average was applied to the sample value. Quantification of mRNA for each sample was calculated below. Serial diluted standard templates (highest = 1) were performed together with experimental samples. Relative initial amplicon concentration to standard was calculated by standard curve of the cycle threshold. Then each sample value was divided with that of GAPDH. Three experimental mean values are shown.

Proliferation assay

The cell number was counted using Cell Counting Kit (DOJINDO) according to the manufacture's protocol. Briefly, 96 MicroWell™ Optical Bottom Plates (NUNC) were coated with collagen type I (10 μg/ml). Cells were cultured for 24 h (1.0×10^4 cells/well) and then incubated with or without 10^{-7} M LTC4 in the 5% or free serum medium containing Serine Borate Complex (5 mM Serine, 10 mM Borate), LTC4 converting inhibitor (22). Cells were incubated with counting solution for 1 h. Absorbance was quantified at 450 nm using Wallac ARVO (PerkinElmer Life and Analytical Sciences, Inc). A receptor antagonist was applied 15 min before the stimulation with CysLTs, and PTX was performed 24 h before.

Calcium response assay

Intracellular calcium response was measured using Calcium Kit Fluo3 (Dojindo) according to the manufacture's protocol. Briefly, the HEK293 cells stably expressing gpCysLT2 (HEKgpCysLT2) were cultured in 96 MicroWell™ Optical Bottom Plates (Nalge Nunc International) for 12 h (5×10^4 cells/well). The culture medium was removed and loading medium containing Fluo3-AM was loaded. After 1 h incubation, the cells were loaded with recording medium. The change in the fluorescence (490 nm) in response to LTC4 or LTD4 was monitored using a Wallac ARVO (PerkinElmer Life and Analytical Sciences, Inc, Wellesley, MA). Intracellular calcium was calculated as $K_d[(F - F_{min})/(F_{max} - F)]$, where K_d is the dissociation constant given a value of 400 nM at 20°C (approximately equal to room temperature) (28). F_{max} was measured after exposing cells to 0.1%w/v TritonX-100 for 10 min. F_{min} was then measured after exposing cells to 10 mM EGTA for 10 min. A receptor antagonist was applied 15 min before stimulation with LTC4 or LTD4.

Acknowledgment/Footnotes

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