Characterization of the Human Cysteinyl Leukotriene 2 Receptor*

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Christopher E. Heise‡‡, Brian F. O’Dowd‡, David J. Figueroa‡, Nicole Sawyer**, Yuan Nguyen‡, Dong-Sooon Im‡, Rino Stooco‡**, Julie N. Bellefeuille**, Mark Abramovitz**, Regina Cheng‡, David L. Williams Jr., Zhizhen Zeng, Qingyun Liu, Lei Ma, Michelle K. Clements‡, Nathalie Coulombe**, Yuan Liu‡, Christopher P. Austin, Susan R. George‡, Gary P. O’Neill**, Kathleen M. Metters**, Kevin R. Lynch‡‡, and Jilly F. Evans‡‡

From the ‡Department of Pharmacology, University of Virginia, School of Medicine, Charlottesville, Virginia 22908, the §Department of Pharmacology, University of Toronto, Medical Sciences Building, Toronto, Ontario MSS 1A8, Canada, Centre for Addiction and Mental Health, Toronto, Ontario MSS 2S1, Canada, the Departments of Pharmacology and ¶¶Bioinformatics, Merck & Co., Inc., West Point, Pennsylvania 19486, and the **Department of Biochemistry and Molecular Biology, Merck Frosst Canada & Co., Pointe-Claire-Dorval, Quebec H8R 4P8, Canada

The contractile and inflammatory actions of the cysteinyl leukotrienes (CysLTs), LTC₄, LTD₄, and LTE₄, are thought to be mediated through at least two distinct but related CysLT G protein-coupled receptors. The human CysLT₁ receptor has been recently cloned and characterized. We describe here the cloning and characterization of the second cysteinyl leukotriene receptor, CysLT₂, a 346-amino acid protein with 38% amino acid identity to the CysLT₁ receptor. The recombinant human CysLT₂ receptor demonstrated high affinity and HEK293T cells and showed to elevated affinity of intracellular calcium when activated by LTC₄, LTD₄, or LTE₄. Analyses of radiolabeled LTD₄ binding to the recombinant CysLT₂ receptor demonstrated high affinity and a rank order of potency for competition of LTC₄ = LTD₄ > LTE₄. In contrast to the dual CysLT₁/CysLT₂ antagonist, BAY u9773, the CysLT₂ receptor-selective antagonists MK-571, montelukast (Singulair™), zafirlukast (Accolate™), and pranlukast (Onon™) exhibited low potency in competition for LTD₄ binding and as antagonists of CysLT₂ receptor signaling. CysLT₂ receptor mRNA was detected in lung macrophages and airway smooth muscle, cardiac Purkinje cells, adrenal medulla cells, peripheral blood leukocytes, and brain, and the receptor gene was mapped to chromosome 13q14, a region linked to atopic asthma.

The cysteinyl leukotrienes (CysLTs), LTC₄, LTD₄, and LTE₄, previously known as slow reacting substance of anaphylaxis, or SRS-A, are derived from arachidonic acid via oxygenation and dehydrogen by 5-lipoxygenase followed by specific glutathione addition by LTC₄ synthase (1). The CysLTs mediate their biological actions through two pharmacologically defined G-protein-coupled receptors (GPCRs), named the CysLT₁ and CysLT₂ (2, 3). The recent cloning and characterization of the human CysLT₁ receptor confirmed the previous pharmacological data (Refs. 4 and 5; GenBank™ accession nos. AF 119711 and AF 133266). LTD₄ is the preferred endogenous ligand for the CysLT₁ receptor, and activation of the receptor results in an elevation of intracellular calcium (4, 5). The gene for the CysLT₁ receptor has been mapped to human chromosome Xq13-q21 (4, 5). The CysLT₁ receptor is the molecular target of the anti-asthmatic drugs montelukast (Singulair™), zafirlukast (Accolate™), and pranlukast (Onon™) that have both anti-bronchoconstrictive and anti-inflammatory actions (6–8). All known CysLT receptor antagonists, except BAY u9773 (a nonselective antagonist at CysLT₁ and CysLT₂ receptors) selectively antagonize activation of the CysLT₁ receptor (6–9). CysLT₁ receptor mRNA and protein are expressed on human lung smooth muscle cells and tissue macrophages and on peripheral blood monocytes and eosinophils (4, 2).

The CysLT₂ receptor has been documented pharmacologically to be expressed in guinea pig trachea and ileum, ferret trachea and spleen, sheep bronchus, and human pulmonary and saphenous vein preparations (2, 10, 11). At the CysLT₂ receptor subtype, the agonist potency rank order is LTC₄ = LTD₄ > LTE₄, and LTE₄ is a partial agonist (2). We describe here the molecular cloning and characterization of the human CysLT₂ receptor.

EXPERIMENTAL PROCEDURES

Materials—LTD₄ and LTC₄ were from Cayman (Ann Arbor, MI); LTE₄, BAY u9773 were from BIOMOL (Plymouth Meeting, PA); 1-oleoyl lysophosphatidic acid was from Avanti Polar Lipids (Alabaster, AL). MK-571, montelukast, pranlukast, and zafirlukast were synthesized by the Department of Medicinal Chemistry at Merck Frosst, and [3H]LTD₄ (146 Ci/mol) was from NEN Life Science Products.

Cloning of HG57, the CysLT₂ Receptor—A partial rat EST (accession no. ai178926) encoding a GPCR fragment, with 40% identity to the human CysLT₁ receptor, was found during a routine search of updates to the GenBank™ data base of expressed sequence tags using the FAST PAN program (12). The cognate cDNA was retrieved from the American Type Culture Collection and was used to screen a rat brain cDNA library as reported previously (13). Two cDNAs were obtained that had a translational open reading frame of 981 nucleotides. This putative receptor, designated by the expressed sequence tags clone name, RSPBT32, was 40% identical to the human CysLT₁ receptor but 16 and 21 amino acids shorter at the N- and C-terminal ends, respect-

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To whom correspondence should be addressed: Dept. of Pharmacology, Merck & Co., Inc., 770 Sumneytown Pike, P.O. Box 4, West Point, PA 19486. Tel.: 215-652-1254; Fax: 215-993-4007; E-mail: jilly.evans@merck.com.

The abbreviations used are: CysLT, cysteinyl leukotriene; LTC₄, leukotriene C₄; LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; SSPE, saline/sodium phosphate/EDTA; GPCR, G-protein-coupled receptor.
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**In Situ Hybridization Analysis and Immunohistochemistry—** The oligonucleotide anti-sense probes used for the *in situ* studies were 5'-C-GGACGACTCTGAGTTC-3' and 5'-CCACCCAGGAGGAA-3', and their complementary sequences were used as sense probes. Tailing of oligonucleotides with biotin-16-dUTP (Roche Molecular Biochemicals) followed the manufacturer's directions (NEN Life Science Products) according to the manufacturer's instructions. Peripheral blood mononuclear cells were isolated from buffy coat preparations by centrifugation over lymphocyte separation medium (ICN). T cells were resuspended by incubation of the peripheral blood mononuclear cells with neuraminidase-treated sheep red blood cells and pelleted through lymphocyte separation medium. The sheep red blood cells were removed by lysis with ACK lysis buffer (Life Technologies). T cell-depleted peripheral blood mononuclear cells accumulated at the interface of the lymphocyte separation medium. Eosinophils were prepared from peripheral blood from a donor known to have elevated peripheral blood eosinophils but no history of asthmatic disease. Erythrocytes were removed by hypotonic lysis of the pelleted cells, followed by negative selection with anti-CD16 microbeads (Milenyi Biotec) according to the manufacturer's instructions. The purity of eosinophil preparations was >90% with some contaminating monocytes and neutrophils. All preparations of cells were resuspended in O.C.T. compound (Miles Scientific) and fresh frozen prior to *in situ* hybridization. **In situ** hybridization on these cell preparations was carried out exactly as described above for tissue sections. Sections and cells were counterstained with 4,6-diamidino-2-phenylindole (Molecular Probes, Inc., Eugene, OR) to visualize cell nuclei, and images were digitally acquired and reassembled using a MicroMax CCD camera (Princeton Instruments) and the Metamorph imaging program (Universal Imaging). Cell nuclei are seen in true blue pseudocolor or in conventional 4,6-diamidino-2-phenylindole blue. Immunohistochemistry was performed after *in situ* hybridization, with avidin (Vector Laboratories Inc.) and biotinylated anti-mouse IgG (Jackson Immunoresearch). A fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson Immunoresearch) or a fluorescein isothiocyanate-conjugated donkey anti-rat IgG (Jackson Immunoresearch). Colocalization of red (in *situ*) and green (antibody) staining is seen as yellow fluorescence.

**Chromosomal Localization—** Chromosomal mapping of the CysLT<sub>2</sub> receptor gene was performed using the GeneBridge 4 panel, consisting of 93 radiation hybrid clones (Research Genetics, Huntsville, AL). Two primer pairs were designed from the HG57 sequence to amplify each of two different regions of the gene. Primer pair 1 sequences (5'-AATGG-CACATTGCAAGAACT-3' (forward) and 3'-AGACGACACCATCC-5') produced a band of ~100 base pairs; primer pair 2 sequences (5'-AGACTGCAAAATGGTTGTTATC-3' (forward) and 5'-ATACCTTGTGTTTCTTACAC-3') (reverse) produced a band of ~180 base pairs. Polymerase chain reaction was performed with AmpliTaq Gold (Perkin-Elmer) using the following cycling parameters: 94° C for 9 min; 94° C for 2 min, 62° C for 30 s, 72° C for 1 min (32 cycles), and 72° C for 7 min. Results were submitted to the Whitehead Institute Genome Center server (available on the World Wide Web) and confirmed by QUANTUM Somatic Cell Hybrid PCRable Panel (QUANUM) and Stanford G3 Radiation Hybrid Panel (Research Genetics). RESULTS AND DISCUSSION

**Sequence Comparison—** Phylogenetic analysis (Fig. 1A) showed that the human orphan GPCR HG57 (identified here as the CysLT<sub>2</sub> receptor) was 73% identical to the rat orphan GPCR RSBPT32 sequence, 38% identical to the human CysLT<sub>1</sub> receptor sequence, and 35% identical to the human orphan GPCRs GPR17 and GPR23. Despite its high similarity to HG57, we have been unable to detect expression of the rat RSBPT32 receptor (data not shown). The deduced amino acid sequence of HG57 (the CysLT<sub>2</sub> receptor), portrayed as a putative seven-transmembrane domain protein in three-dimensional helical representation, is shown in Fig. 1B (GenBank<sup>TM</sup> sequence AF254664). The primary sequence of the CysLT<sub>2</sub> receptor is quite dissimilar to another leukotriene re-
ceptor, the BLT high affinity LTB4 receptor (Ref. 20; Fig. 1A).

Although there is no record of HG57 in the expressed sequence
tags division of GenBankTM, an identical open reading frame is
contained as an HTGS deposition (accession no. AL137118).

Xenopus Oocyte Functional Activation—The CysLTs LTD4
and LTC4 produced calcium-dependent chloride flux in HG57
(CysLT2) receptor cRNA-injected X. laevis oocytes and were
desensitized to subsequent challenge by CysLTs (Fig. 2A and
data not shown). Control oocytes injected with saline or other
GPCR cRNAs, including those encoding RSPBT32 and GPR17,
showed no response to LTD4 or LTC4 but were able to respond
to lysophosphaticidic acid challenge through an endogenous ly-
sophosphaticidic acid receptor (Fig. 2B). LTD4 and LTC4 pro-
duced dose-dependent activation of calcium-dependent chloride
flux in HG57 (CysLT2) receptor cRNA-injected oocytes (n = 3–8)
to 1 μM LTD4 pretreated with MK-571 or BAY u9773.

Transient Expression and Functional Activation in Mamma-
lian Cells—In HG57 (CysLT2) and aequorin-co-expressing
HEK293T cells, LTD4 and LTC4 were equipotent agonists,
while LTE4 behaved as a partial agonist, and LTB4 was inac-
tive up to 3 μM (Fig. 3A). These agonists show no response in
vector-transfected aequorin-expressing HEK293T cells (data
not shown). BAY u9773 acted as a noncompetitive antagonist of
both LTD4 and LTC4 challenged CysLT2 receptor-expressing
HEK293T cells (Fig. 3B). The selective CysLT1 receptor antag-
onists, MK-571, montelukast, zafirlukast, and pranlukast,
showed no significant antagonism of the CysLT2 receptor up to
1 μM concentration (Fig. 3C).

Radioligand Binding Characterization—Saturation analysis of
[^3H]LTD4-specific binding to COS-7 cell membranes trans-
iently expressing HG57 (CysLT2) was performed in two sepa-
rate experiments with two different membrane preparations.

**Fig. 1. Amino acid homologies and sequence representation of
HG57, the human CysLT2 receptor.** A, phylogenetic tree represent-
ing amino acid sequence similarities between the human CysLT2
(HG57) receptor (accession no. AF254664) and related GPCRs from
family 1. These include CysLT1_hum (AF119711), RSPBT32_rat
(AI178926), GPR17_hum (U33447), P2Y1_hum (Z49205), P2Y2_hum
(U07225), P2Y6_hum (X97058), HG12_hum (af_000545), RB_intron-
hum (L11910), GPR23_hum (U66578), and BLT (LTB4)_hum
(U41070). The Wisconsin Sequence Analysis Package (version 10.0) was
used in the analysis. Branch lengths along the x axis are inversely
proportional to percentage of amino acid identity. B, three-dimensional
representation of the amino acid sequence of the CysLT2 receptor.
Glycosylation and phosphorylation sites are indicated, as are the puta-
tive seven-transmembrane helices. Amino acids identical in the CysLT1
and CysLT2 receptors are indicated by red circles.
In one case (Fig. 4A), data analysis using Prism (GraphPad Software Inc.) revealed the presence of high and low affinity binding sites (Kd 50.4 and 51 nM; Bmax 50.135 and 1.415 pmol/mg of membrane protein, respectively) as illustrated in the Scatchard representation of the deduced specific binding isotherm (Fig. 4A, inset). However, in the second case, saturation analysis showed the presence of a single population of binding sites (Kd 54.8 nM; Bmax 50.338 pmol/mg of membrane protein). This difference is potentially due to variations between membrane preparations inherent in using a transient expression system. We are currently developing clonal cell lines expressing CysLT2 that should permit a more accurate assessment of ligand:receptor affinity and receptor number. In equilibrium competition assays, the rank order of potency of leukotriene agonists to compete with [3H]LTD4 for binding to the CysLT2 receptor was LTD4 > LTC4 > LTE4 with no competition up to 10 μM by LTB4 (Fig. 4B). CysLT1 receptor antagonists were either weak (zafirlukast and pranlukast) or inactive.

**Fig. 4.** Radioligand binding studies with COS-7 cell membranes expressing HG57, the human CysLT2 receptor. A, saturation analysis of [3H]LTD4 binding to HG57 (CysLT2). Radioligand binding assay was performed in the presence of increasing amounts of [3H]LTD4 supplemented with unlabeled ligand (final specific activity of [3H]LTD4 = 26.8 Ci/mmol) over a range of concentration of 0.04–33 nM total LTD4. The calculated [3H]LTD4-specific binding saturation isotherm was analyzed by nonlinear transformation using Prism (GraphPad Software Inc.) to generate Kd and Bmax values. A representative saturation curve from two independent experiments done in duplicate with two different membrane preparations is shown. Inset, Scatchard representation of the specific binding isotherm. ○, total; □, nonspecific; ●, specific. B, equilibrium competition assays for [3H]LTD4-specific binding to HG57 (CysLT2) with leukotrienes and CysLT-specific antagonists. Representative titration curves from at least two independent experiments done in duplicate are shown. Competition curves were analyzed with a custom designed software using a nonlinear least-squares curve fitting routine based on a four-parameter logistic equation to determine half-maximal inhibitory concentration (IC50) values. ▽, LTC4; ●, LTD4; ▲, LTE4; ◇, LTB4; ○, BAY u9773; ×, montelukast; ●, zafirlukast; □, pranlukast.

In one case (Fig. 4A), data analysis using Prism (GraphPad Software Inc.) revealed the presence of high and low affinity binding sites (Kd = 4.8 nM; Bmax = 0.338 pmol/mg of membrane protein), respectively. This difference is potentially due to variations between membrane preparations inherent in using a transient expression system. We are currently developing clonal cell lines expressing CysLT2 that should permit a more accurate assessment of ligand:receptor affinity and receptor number. In equilibrium competition assays, the rank order of potency of leukotriene agonists to compete with [3H]LTD4 for binding to the CysLT2 receptor was LTD4 > LTC4 > LTE4 with no competition up to 10 μM by LTB4 (Fig. 4B). CysLT1 receptor antagonists were either weak (zafirlukast and pranlukast) or inactive.
(montelukast) at competing for radiolabeled LTD₄ binding to the CysLT₂ receptor (Fig. 4B and Table I). However, full competition was observed with the dual CysLT₁/CysLT₂ antagonist BAY u9773 with an IC₅₀ value of 0.6 μM (Fig. 4B and Table I). COS-7 cell membranes transfected with vector alone showed no specific LTD₄ binding, as previously reported (4).

**Human Tissue RNA Northern Blot and in Situ Analyses**—

The human CysLT₂ receptor was shown to be expressed in peripheral blood leukocytes, lymph node, spleen, heart, and several central nervous system regions (Fig. 5). In human RNA dot blot analyses, the receptor was also shown to be expressed in the adrenal gland (data not shown). The CysLTs are known to have potent contractile and inflammatory effects in the human lung, so we investigated the in situ expression of...
CysLT₂ receptor mRNA in human lung (Fig. 6, A–E). In contrast to the CysLT₁ receptor, which was most highly expressed in human lung smooth muscle (4), the strongest lung expression of the CysLT₂ receptor was seen in interstitial macrophages (Fig. 6, A–C) with distinctly weaker expression in smooth muscle cells (Fig. 6, D and E). It was of interest that we noted particularly elevated expression of the CysLT₂ receptor in macrophages when in close proximity to smooth muscle cells (Fig. 6D). Peripheral blood leukocytes were another abundant source for CysLT₂ receptor transcripts (Fig. 5). We carried out in situ analysis of the CysLT₂ receptor in partially purified normal human peripheral blood monocytes and demonstrated abundant expression in greater than 20% of these cells (Fig. 6F). In addition, we found strong expression of the receptor in purified human eosinophils, a cell type in which we had previously shown expression of the CysLT₂ receptor (Ref. 4; Fig. 6, G and H).

Tissues other than myeloid cell containing organs, such as the spleen and lymph nodes, that showed expression of the mRNA for the CysLT₂ receptor included regions of the central nervous system, the adrenal gland, and the heart. The synthesis and activities of cysteinyl leukotrienes in the brain of various animal species has been well documented (21–23). Studies on expression of both the CysLT₁ and CysLT₂ receptors in the cardiovascular, endocrine, and central nervous system tissues.

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REFERENCES

1. Samuelsson, B. (1983) Science 220, 568–575
2. Coleman, R. A., Eglen, R. M., Jones, R. L., Narumui, S., Shimizu, T., Smith, W. L., Dahlén, S. E., Durieux, M. E., Gardiner, P. J., Jackson, W. T., Jones T. R., Krell, R. D., and Nicolas, S. (1995) Adv. Prostaglandin Thromboxane Leukotriene Res. 23, 283–285
3. Metters, K. M. (1995) J. Lipid Mediat. Cell Signal. 12, 411–427
4. Lynch, K. R., O’Neill, G. A., Liu, Q., Im, D.-S., Sawyer, N., Metters, K. M., Courouble, N., Abramovitz, M., Figuera, D. J., Zeng, Z., Connolly, B. M., and Samuelsson, B. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 318–326
5. Suissa, S., Dennis, R., Ernst, P., Sheehy, O., and Wood-Dauphine, S. (1997) Ann. Int. Med. 126, 177–183
6. Grossman, J., Faierman, I., Duhl J. W., Tompson, D. J., Busse, W. B., Bronsky, E., Montanaro, A., Southern, L., and Tinkelman, D. (1997) J. Asthma 34, 321–328
7. Tudhope, S. R., Cuthbert, N. J., Abram, T. S., Jennings, M. A., Maxey, R. J., Thompson, A. M., Norman, P., Gardiner, P. J. (1994) Eur. J. Pharmacol. 264, 317–323
8. 2
9. Labat, C., Ortiz, J. L., Norel, X., Gascard, J. P., and Samuelsson, B. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 318–326