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Molecular cloning and functional characterization of murine cysteinyl-leukotriene 1 ($CysLT_1$) receptors

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Abstract

We sought to clone and characterize the murine cysteinyl-leukotriene D_4 receptor ($mCysLT_1R$) to complement our studies with leukotriene-deficient mice. A cDNA, cloned from trachea mRNA by reverse transcriptase–polymerase chain reaction, has two potential initiator ATG codons that would encode for polypeptides of 352 and 339 amino acids, respectively. These two potential forms, predicted to be seven transmembrane-spanning domain proteins, have 87% amino acid identity with the human $CysLT_1$ receptor ($hCysLT_1R$). Membrane fractions of Cos-7 cells transiently expressing the short $mCysLT_1R$ demonstrated high affinity and specific binding for leukotriene D_4 (LTD_4, $K_d = 0.25 \pm 0.04$ nM). In competition binding experiments, LTD_4 was the most potent competitor ($K_i = 0.8 \pm 0.2$ nM) followed by LTE_4 and LTC_4 ($K_i = 86.6 \pm 24.5$ and 100.1 ± 17.1 nM, respectively) and LTB_4 ($K_i > 1.5$ μM). Binding of LTD_4 was competitively inhibited by the specific $CysLT_1$ receptor antagonists MK-571 [(+)-3-(((3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl) ((3-(dimethylamino)-3-oxopropyl)thio)methyl)thio)propanoic acid], pranlukast (Onon™), and zafirlukast (Accolate™), while the $CysLT_1$/$CysLT_2$ receptor antagonist BAY-u9773 [6(*R*)-(4′-carboxyphenylthio)-5(*S*)-hydroxy-7(E),9(E),11(Z),14(Z)-eicosatetrenoic acid] was 1000 times less potent than LTD_4. In transiently transfected HEK293-T cells expressing either the long or short form of $mCysLT_1R$, LTD_4 induced an increase of intracellular calcium. In *Xenopus laevis* melanophores transiently expressing either isoform, LTD_4 induced the dispersion of pigment granules, consistent with the activation by LTD_4 of a $G_{\alpha q}$ (calcium) pathway. Functional elucidation of $mCysLT_1R$ properties as described here will enable further experiments to clarify the selective role of LTD_4 in murine models of inflammation and asthma.

Keywords: Leukotriene; Asthma; Receptor; Murine; GPCR; Antagonist

1. Introduction

LTs are lipid mediators generated *de novo* from the ubiquitous membrane-component arachidonic acid. Cysteine-containing leukotrienes, LTC_4, LTD_4, and LTE_4, also known as slow-reacting substance of anaphylaxis (SRS-A), are considered to be key mediators in the pathophysiology of different inflammatory and allergic disorders, particularly in asthma.

Asthma is a chronic multifactorial lung disease distinguished by a variety of sporadic episodes of wheezing, shortness of breath, mucus production, and cough. The occurrence of reversible airway obstruction (narrowing of the airways diameter), thick mucus secretion, edema of the bronchial wall, inflammation, and airway hyper-reactivity to a variety of specific or non-specific stimuli [1] characterize this disorder.

Murine models of allergic inflammation that mimic some aspects of the asthmatic response are commonly used in asthma research. We have shown previously that LT-deficient mice generated by targeted disruption of the 5-lipoxy-

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Abbreviations: LT, leukotriene; $hCysLT_1R$, human cysteinyl-leukotriene receptor (subtype 1); $mCysLT_1R$, mouse cysteinyl-leukotriene receptor (subtype 1); $CysLT_2R$, cysteinyl-leukotriene receptor (subtype 2); HEK, human embryonic kidney; ORF, open reading frame; RT–PCR, reverse transcription–polymerase chain reaction; and RACE, rapid amplification of cDNA ends.

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genase gene have diminished numbers of eosinophils recruited into the airways and are markedly less hyperresponsive to methacholine challenge [2]. However, mice respond differently than humans to LT challenge [3]. Therefore, it is important to characterize these differences at the receptor level to be able to extend findings from murine "asthma-like" models to humans.

Cysteinyl-leukotrienes mediate their effects through at least two G protein-coupled receptors, $CysLT_1$ and $CysLT_2$ [4–7]. Most of the biological properties relevant to asthma appear to be mediated through $CysLT_1$ receptor stimulation. Using drugs to inhibit the synthesis of cysteinyl-leukotrienes or to antagonize their effects mediated by $CysLT_1$ receptor activation in the airways significantly diminishes allergen-, aspirin-, and exercise-induced broncho-constriction [8,9]. Two clinical anti-LT drugs, zafirlukast (Accolate™) and montelukast (Singulair™), both antagonists of the $CysLT_1$ receptor, available in the United States for the past 4–5 years, have proven useful in the management of mild-moderate asthma. However, there has been a paucity of new information on receptor signaling until the recent molecular cloning of human $CysLT_1$ [10,11] and $CysLT_2$ [12–14] receptors.

As a prelude for the exploration of the effects of cysteinyl-leukotrienes mediated through the $CysLT_1$ receptor in mouse inflammation models such as induced-asthma, we have cloned and pharmacologically characterized the murine $CysLT_1$ receptor.

2. Materials and methods

2.1. Materials

LTD_4, LTC_4, LTE_4, and LTB_4 were purchased from Cayman Chemical. BAY-u9773 [6(*R*)-(4′-carboxyphenylthio)-5(*S*)-hydroxy-7(E),9(E),11(Z),14(Z)-eicosatetrenoic acid] was purchased from Biomol. MK-571 [(+)-3-(((3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl) ((3-(dimethylamino)-3-oxopropyl)thio)methyl)thio)propanoic acid], pranlukast (Onon™), and zafirlukast (Accolate™) were synthesized in the Department of Medicinal Chemistry at Merck Frosst. [^{3} H]LTD_4 was from NEN Life Science Technology.

2.2. Cloning of mouse $CysLT_1$ receptor cDNA

Degenerate oligonucleotide primers were designed based on the human $CysLT_1$ receptor sequence (amino acids 63–71 and 291–299) and mouse and human lipoxin A_4 receptor sequences in these corresponding regions: upstream 5′-ATHAACYTNGCAGTNGCACATCTAC-3′ and downstream 5′-CCCRGARAARAAATATAGNAGAGGGTC-3′. These primers were used in RT–PCR reactions with mouse trachea cDNA template. A ≈600 bp fragment was cloned and sequenced. Since only a partial length clone was isolated, RT–PCR with rapid amplification of cDNA 5′ and 3′ ends (RACE method) was performed.

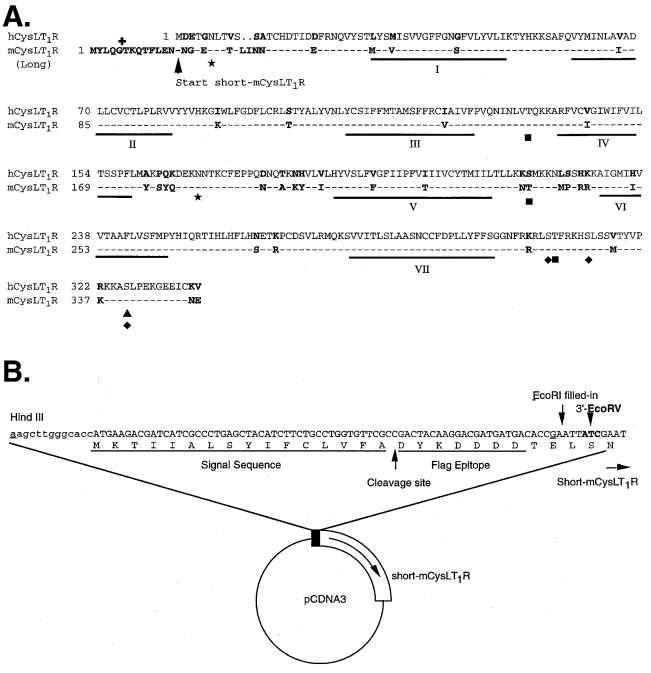

Fig. 1. (A) Predicted amino acid sequence of mouse $CysLT_1$ receptors compared with that of its human ortholog. Dashes indicate amino acids conserved between mouse and human receptors. Residues different between the mouse and human receptors are denoted in bold. The putative seven transmembrane domains are underlined and numbered. Key: (★) putative *N* -glycosylation sites; (◆) putative cAMP-, cGMP-protein kinase; (■) putative protein kinase C; and (▲) putative casein kinase 2 phosphorylation sites. The cross denotes a potential myristoylation site in the extracellular N-terminal region of the long-mCysLT$_1$ R, if suitable N-terminal processing were to take place. The GenBank Accession Number for mCysLT$_1$ R is AF205830. (B) Diagram of the plasmid encoding the influenza hemagglutinin signal sequence-FLAG epitope-tagged mCysLT$_1$ R (short-FLAG-mCysLT$_1$ R) used in most binding and functional experiments. See "Materials and methods" for a description of the construct.

The ORFs were amplified by PCR using upstream primers CDF542 for the long isoform (5′-GTAGGTACCGCCATGTACCTCCAAGGC-3′) or CDF543 for the short isoform (5′-GTAGGTACCATGAATGGAACTGAAAATC-3′) and downstream primer CDF544 (5′-GACGGATCCTTATTCGTTACATATTTCTTC-3′). The sequences of the amplified products were verified, digested with *Kpn*I and *Bam*HI (New England BioLabs) and cloned into *Kpn*I-*Bam*HI digested pCDNA3 (Invitrogen) expression vector. The constructs obtained were designated as long-mCysLT$_1$R and short-mCysLT$_1$R, respectively.

The short isoform produced by PCR with CDF 556 (5′-GGATATCGAATGGAACTGAAAATCTGACG-3′) and CDF 557 (5′-GCTCTAGATTATTCGTTACATATTTCTTCTC-3′), digested by *Eco*RV-*Xba*I, was cloned in a pCDNA3-FLAG vector containing an influenza hemagglutinin signal sequence (gift from Dr. J. Benovic, Thomas Jefferson University) [15]. The construct was designated as short-FLAG-mCysLT$_1$R (see construct, Fig. 1B).

2.3. Cell culture and transfection

Mammalian monkey kidney Cos-7 cells or human embryonic kidney (HEK) 293 cells stably expressing the SV40 large T antigen, designated HEK293-T cells, were maintained in Dulbecco's modified Eagle's medium containing 25 mM HEPES, 100 U/mL of penicillin and 100 μg/mL of streptomycin, 1 mM sodium pyruvate supplemented with 10% (v/v) fetal bovine serum in a 6% CO_2/air incubator at 37°. HEK293-T cells were maintained in the presence of 250 μg/mL of G418. Cells were transfected with LipofectAMINE™ PLUS reagent (Gibco-BRL) following the instructions of the manufacturer.

2.4. Radioligand binding experiments

Tritiated LTD_4-binding assays were performed as described [16] with the exception that the competitive reactions were initiated by the addition of 30 μg of membrane protein. [^{3}H]LTD_4 specific binding was calculated by subtracting non-specific binding from total binding. Non-specific binding, determined in the presence of MK-571 (10 μM), accounted for ≈10% of the total binding for membranes obtained from short-FLAG-mCysLT$_1$R transfected cells. Average total and non-specific binding values were 3270 and 310 dpm, respectively.

2.5. Aequorin luminescence functional assay

Cos-7 or HEK293-T cells transfected with either short- or long-mCysLT$_1$R, short-FLAG-mCysLT$_1$R, or pCDNA3 and AEQ-pCDM (expressing aequorin) were prepared for use in the aequorin luminescence functional assay as described previously [17]. Briefly, confluent culture cells were charged for 1 hr at 37° in modified Ham's F12 medium (containing 0.1% fetal bovine serum, 25 mM HEPES at pH 7.3) with 30 μM reduced glutathione and 8 μM coelenterazine cp (Molecular Probes). Then cells were washed, harvested with a non-enzymatic dissociation solution (Gibco-BRL, Cat. No. 13151–014), spun down and washed two times sequentially, resuspended in modified Ham's F12, and maintained at room temperature. Agonists, in ethanol, were serially-diluted, in duplicate, in a white 96-well cliniplate FB (Labsystems) in 100 μL final volume. Cells (5×10^5/mL; 100 μL) were injected into each well and mixed, and the emitted luminescence was recorded immediately over 30 sec after which cells were lysed by injection of Triton X-100 (25 μL of 0.9% solution), and light emission was measured for an additional 10 sec. Fractional luminescence for each well was determined by dividing the area under the first peak (luminescence triggered by the agonist) by the total area under peaks 1 and 2 (luminescence triggered by Triton X-100). The EC_{50} values were calculated using a modified version of the Levenberg-Marquardt four-parameter curve-fitting algorithm [17]. Vehicle (EtOH) was < 1% of the final volume and did not elicit a detectable response.

2.6. Melanophore functional bioassay

Xenopus laevis melanophores were maintained in culture and used as described previously [18–20]. Briefly, transient expression of short-FLAG-mCysLT$_1$R in melanophores was achieved by electroporation. Melanophores were plated (15,000/well) in 96-well tissue culture plates and cultured for 2 days. Prior to the addition of agonist, cells were washed, incubated for 1 hr with 0.7× L-15 medium (Gibco-BRL Cat. No. 11415–064) containing 10 nM melatonin to pre-aggregate the melanosomes [18]. The melatonin activates an endogenous G_i coupled receptor, which causes pigment aggregation. Baseline absorbance (A_0) reading was obtained at 620 nm using a Molecular Devices V_{max} kinetic microtiter-plate reader. The agonist LTD_4 (or LTC_4) was added to the microtiter wells in 20-μL aliquots at 10 times their final concentration. Concentration–response data were obtained 1 hr later (A_f). Data were plotted with $y = 1 - (A_f/A_0)$. Data are presented as means ± SEM.

3. Results

3.1. Cloning and sequencing of mouse CysLT$_1$ receptor cDNA

At the onset of this project, one human CysLT$_1$R sequence had been reported [10] and no CysLT$_2$R sequences were cloned. Thus, degenerate primers were designed based on transmembrane domains II and VII sequences of the human CysLT$_1$ and human/mouse lipoxin A_4 receptors. PCR was performed on cDNA from mouse trachea, a tissue known to express CysLT$_1$ receptors. A ≈600 bp cloned fragment showed about 80% sequence homology with the hCysLT$_1$ receptor. Subsequent 3′- and 5′-RACE reactions using this fragment yielded products encompassing 1521 bp with the longest predicted ORF of 1059 bp (GenBank Accession Number: AF205830). This particular ORF encodes a 352 amino acid protein (designated long-mCysLT$_1$R) with seven putative transmembrane domains typical of G protein-coupled receptors. Alignment of this sequence with the hCysLT$_1$R sequence indicates that the murine form is 13 residues longer at the N-terminus (Fig. 1A). Additional cloning of the CysLT$_1$ gene indicates a splice site between the first and second ATG (Funk CD, unpublished observations). This second methionine, which could represent a potential initiator codon, is aligned precisely with the hCysLT$_1$R initiator codon. Starting from this second initiator codon, the nucleotide sequence would encode a shorter 339-residue protein (designated short-mCysLT$_1$R) similar in size to the human receptor (337 amino acids). Analysis of the sequences surrounding the initiator codons (GAA*ATG*T) and (AAC*ATG*A) for the long and short forms of the mCysLT$_1$ receptor, respectively, indicates that neither matches the Kozak consensus sequence (RCC*ATG*G) perfectly.

Comparison of the human and the mouse CysLT$_1$ recep-

tor sequences revealed 87% identity at the amino acid sequence level. Long-mCysLT$_1$R displayed a potential *N*-myristoylation site by the NH_2 terminus if appropriate proteolytic processing were to take place. Both isoforms have two putative *N*-glycosylation sites, one close to the NH_2 terminus and a second on the putatitve second extracellular loop in addition to multiple potential phosphorylation sites. The putative *N*-glycosylation and phosphorylation sites conserved between both species are shown in Fig. 1A.

3.2. Radioligand binding experiments

Preliminary binding experiments were done with membrane fractions of Cos-7 cells transiently transfected with either long- or short-mCysLT$_1$R. [^{3}H]LTD$_4$ bound specifically to both isoforms, and the CysLT$_1$R antagonists MK-571, pranlukast, and zafirlukast competed for binding. However, the level of receptor expression was very low (<50 fmol/mg protein), and the non-specific binding was between 30 and 50% of total binding (Fig. 2A). At the same time, aequorin luminescence assays, based on the capability of aequorin to release photons in response to intracellular calcium flux, were performed with Cos-7 cells transiently transfected with either the long- or short-mCysLT$_1$R and compared with hCysLT$_1$R to check their functionality. LTD$_4$ induced an increase in intracellular calcium in Cos-7 cells transfected with either form of mCysLT$_1$R (Fig. 2B).

Next we introduced an NH_2-terminal cleavable membrane insertion signal sequence from influenza hemagglutinin, coupled with the "FLAG" epitope, to the N-terminus of the short isoform of mCysLT$_1$R (short-FLAG-mCysLT$_1$R) to enhance expression and function [21] (see construct, Fig. 1B). As a result, [^{3}H]LTD$_4$ total binding on membrane fractions of Cos-7 cells transiently transfected with short-FLAG-mCysLT$_1$R was increased more than five times compared with Cos-7 cells transiently transfected with short-mCysLT$_1$R (Fig. 2A). Moreover, the non-specific binding did not increase (Fig. 2A). The maximal response induced by LTD$_4$ monitored in the aequorin assay was also increased dramatically, and the EC_{50} value decreased approximately 10-fold (Fig. 2C), which is consistent with data showing that enhanced receptor expression can shift the EC_{50} value leftward as well as increase the efficacy of an agonist [22]. Consequently, further experiments were performed using short-FLAG-mCysLT$_1$R.

Saturation analysis of [^{3}H]LTD$_4$ binding to membrane fractions prepared from Cos-7 cells transiently expressing short-FLAG-mCysLT$_1$R demonstrated the presence of high-affinity [^{3}H]LTD$_4$ binding sites (Fig. 3A), with reversible and saturable binding for LTD$_4$ with a $K_d = 0.25 \pm 0.04$ nM and a maximal number of specific binding sites (B_{max}) of 702 ± 22 fmol/mg protein (N = 3). However, the existence of additional lower affinity binding sites cannot be excluded. Displacement curves of [^{3}H]LTD$_4$ binding indicated that the most potent competitor was LTD$_4$ with a K_i of 0.8 ± 0.2 nM (N = 3), followed by LTE$_4$ and LTC$_4$ with K_i

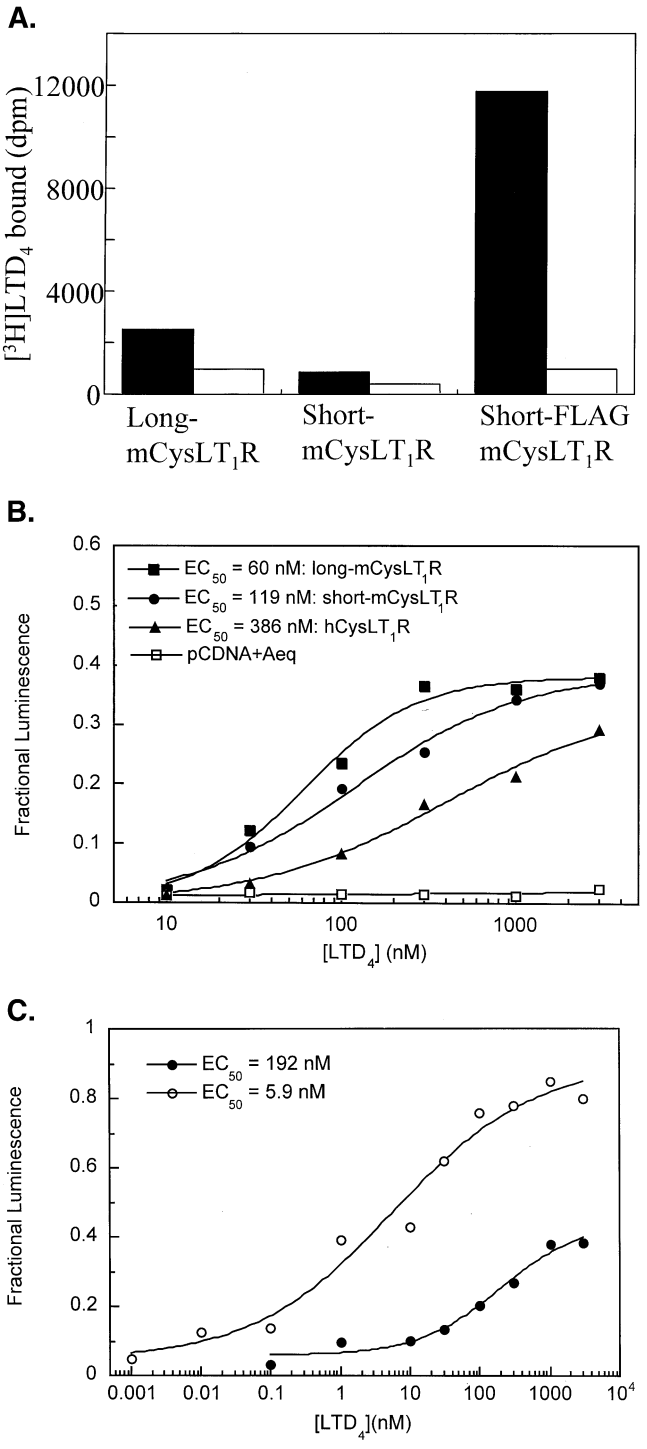

Fig. 2. (A) Total (closed columns) and non-specific [^{3}H]LTD$_4$ (open columns) binding to membrane fractions of Cos-7 cells transiently transfected with long-mCysLT$_1$R (400 μg protein), short-mCysLT$_1$R (400 μg protein), and short-FLAG-mCysLT$_1$R (250 μg protein). A representative experiment of two is shown, and the standard deviation was within 200 dpm for all points. (B and C; N = 2) Aequorin luminescence functional assay. Cos-7 cells were transiently co-transfected with AEQ-pCDM and in panel B, with long-mCysLT$_1$R (■), short-mCysLT$_1$R (●), hCysLT$_1$R-pCDNA3 (▲) or empty vector (□), pCDNA3; in panel C, with short-mCysLT$_1$R (●) or short-FLAG-mCysLT$_1$R (○). The fractional luminescence measured upon stimulation by increasing concentrations of LTD$_4$ is reported as described in "Materials and methods."

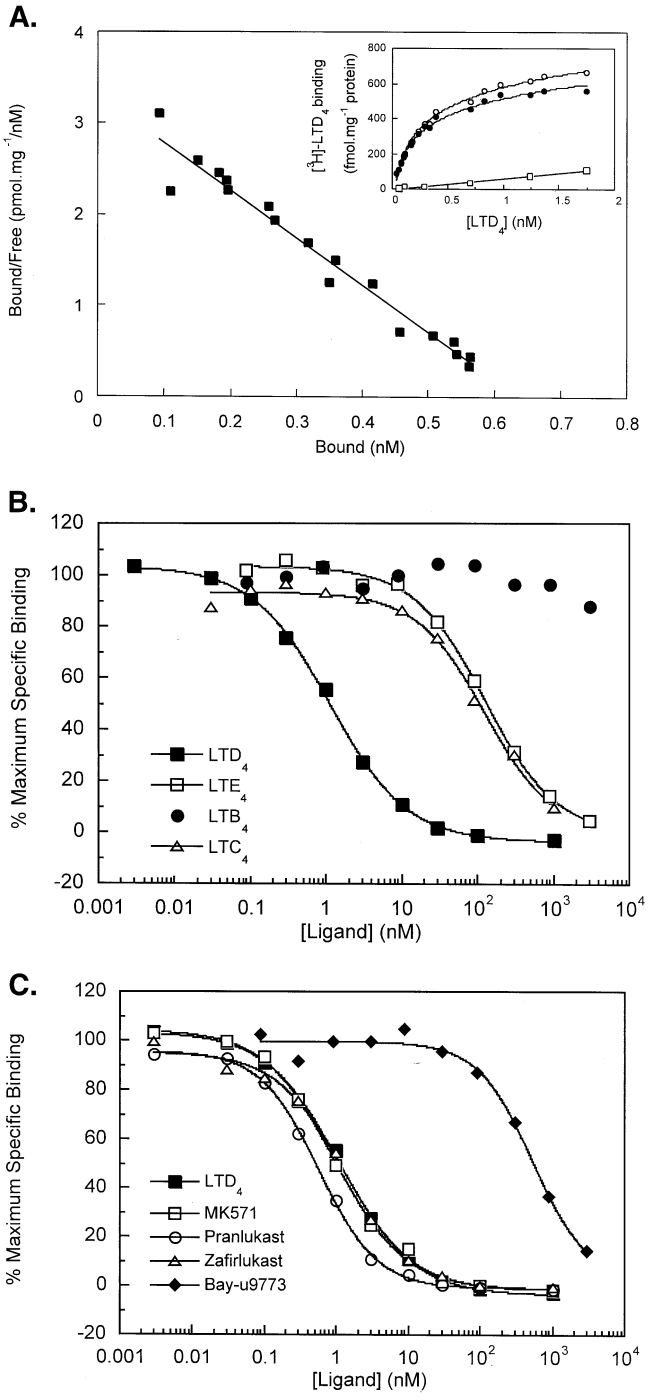

Fig. 3. [^{3}H]LTD_4 binding to membrane fractions of Cos-7 cells transiently transfected with short-FLAG-mCysLT$_1$ R. (A) Scatchard analysis and saturation isotherms of [^{3}H]LTD_4 binding to membrane fractions of Cos-7 cells transiently transfected with short-FLAG-mCysLT$_1$ R. Data from a representative experiment of three giving similar results are shown. The linear Scatchard plot gives a correlation coefficient of 0.98. In this particular experiment, B_{max} and K_d values were 668 fmol/mg protein and 0.27 nM, respectively. The total (○), specific (●), and non-specific (□) binding isotherms are drawn (inset). (B and C) Displacement curves of specific [^{3}H]LTD_4 binding from a representative experiment (N = 3 or 4) are shown with unlabeled LTD_4 (■), LTC_4 (△), LTE_4 (□), and LTB_4 (●) in panel B and with four different antagonists: MK-571 (□), pranlukast (○), zafirlukast (△), and BAY-u9773 (◆) in panel C.

Table 1
LTD_4-induced calcium flux assessed by the aequorin luminescence assay

	EC_{50} (nM)
long-mCysLT$_1$R	59 ± 11
short-mCysLT$_1$R	134 ± 44
hCysLT$_1$R	182 ± 41

The potency of LTD_4 to induce increases in intracellular calcium in Cos-7 cells transiently transfected with either the long- or the short-mCysLT$_1$R or with hCysLT$_1$R was measured.

Values are means ± SEM, N = 4.

values of 86.6 ± 24.5 nM (N = 3) and 100.1 ± 17 nM (N = 4), respectively, while LTB_4 was a very weak competitor ($K_i > 1.5$ μM) (Fig. 3B). CysLT$_1$ receptor specific antagonists MK-571, pranlukast, and zafirlukast displayed high-affinity competitive binding to the short-FLAG-mCysLT$_1$R with pranlukast (K_i = 0.25 ± 0.02 nM, N = 3) ≥ MK-571 (K_i = 0.69 ± 0.12 nM, N = 4) = zafirlukast (K_i = 0.72 ± 0.09 nM, N = 4). The dual CysLT$_1$/CysLT$_2$R antagonist BAY-u9773 was 500-1000 times less potent (K_i = 451 ± 196 nM, N = 3) than the specific CysLT$_1$R antagonists (Fig. 3C).

3.3. Functional assays

The CysLT$_1$R is known to signal through increases of intracellular calcium. We used two different functional systems to characterize the mouse clones.

3.3.1. Aequorin luminescence assay

LTD_4 induced a concentration-dependent calcium flux in either long- or short-mCysLT$_1$R transfected Cos-7 cells, as mentioned above, and its potency was slightly greater than with hCysLT$_1$R transiently transfected Cos-7 cells (Table 1, Fig. 2B). Control cells or Cos-7 cells transfected with pCDNA3 showed no response to LTD_4.

Calcium flux in HEK293-T cells transiently transfected with the short-FLAG-mCysLT$_1$R was concentration-dependently activated by LTD_4 (ec_{50} = 4.9 ± 0.9 nM; N = 4). LTC_4 and LTE_4 were 50 times less potent than LTD_4 (EC_{50} = 188 ± 25 and 225 ± 4 nM, respectively; N = 4; Fig. 4A). LTB_4 did not induce any response up to 3 μM, and preincubation with either MK-571 or pranlukast increased the ec_{50} of LTD_4-induced calcium rise and decreased its maximal effect (Fig. 4B and data not shown).

3.3.2. X. laevis melanophores

X. laevis melanophores respond to agonist-activated endogenous G protein-coupled receptors (GPCRs) by intracellular dispersion of their melanosomes, due to intracellular calcium or cyclic AMP (cAMP) elevation by $G_{\alpha q}$ or $G_{\alpha s}$ activation, and the cells appear dark. Alternatively, aggregation of the melanosomes, due to a decrease in cAMP production by $G_{\alpha i}$ coupling, yields cells that appear light

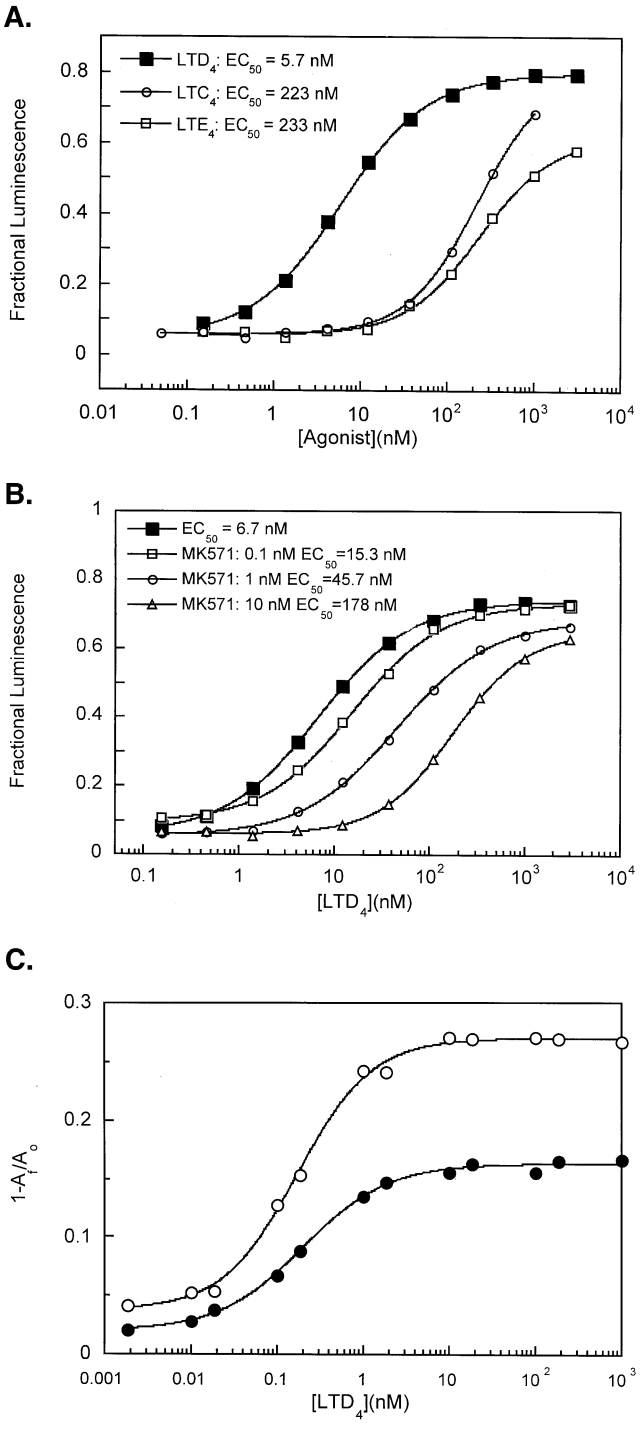

Fig. 4. Functional characterization of mCysLT$_1$R expressed in HEK293-T cells using an aequorin assay (A and B) and in *X. laevis* melanophores (C). Panels A and B: the short-FLAG-mCysLT$_1$R was transfected together with pCDM-Aeq into HEK293-T cells as described in "Materials and methods." Representative fractional luminescence response curves to increasing concentrations of the agonists LTD_4 (■), LTC_4 (○), LTE_4 (□) (panel A), or of LTD_4 in the absence (■) or presence of 0.1 nM (□), 1 nM (○), and 10 nM (△) MK-571 (panel B) are shown (N = 4). Panel C: A representative graph (N = 2) showing mCysLT$_1$R-induced pigment dispersion in melanophores. The long-mCysLT$_1$R (○) and short-mCysLT$_1$R (●) were transfected into *Xenopus* melanophores via electroporation. LTD_4 (2 pM–1 μM) was added to cells exposed to melatonin for 1 hr. The ordinate scale is explained in "Materials and methods."

[19,20]. *X. laevis* melanophores were transfected with long- and short-mCysLT$_1$R. LTD_4 stimulation of both the long- and short-CysLT$_1$R caused pigment dispersion in a concentration-dependent fashion with an EC_{50} value of 0.2 nM for both forms (Fig. 4C). However, the maximum response displayed by the short isoform was lower (Fig. 4C).

4. Discussion

We report here the cloning of murine CysLT$_1$R cDNA encoding for two potential receptor forms differing in the size of their amino terminus: a long form of 352 residues, and a shorter form of 339 residues similar in length to hCysLT$_1$R. These receptors belong to the superfamily of G protein-coupled receptors with seven putative transmembrane-spanning domains. The 13 "extra" amino acid sequence in the long murine form, not present in the human sequence, has no homology to other proteins and no distinguishing features that would indicate any special function. Based on the mCysLT$_1$R gene cloning (Funk *et al.*, unpublished data), this long form could represent a potential splice variant.

Both the long- and short-mCysLT$_1$ receptors bind LTD_4 and are functional. However, due to significant problems with their low-level expression, we focused our attention on a membrane-targeted, FLAG-tagged short-mCysLT$_1$R construct that expressed well and was more amenable to detailed pharmacological and signaling studies. The CysLT$_1$R sequences are very hydrophobic, and a low-level expression has been reported for the human receptor [10]. The influenza hemagglutinin signal/FLAG sequence likely enhances proper processing and membrane insertion as previously described [21] and also perhaps the efficiency of G protein coupling. Perhaps other accessory proteins like RAMPs (receptor activity modifying proteins) [23] are necessary for correct *in vivo* processing and functional expression in bronchial smooth muscle and hematopoietic cells.

LTD_4 binding affinity for the short-mCysLT$_1$R was similar to that for the hCysLT$_1$R ortholog (0.25 vs 0.3 nM, respectively) and comparable to the binding affinities of LTD_4 of human, guinea pig, and rat lung tissues, as well as sheep trachea and lung parenchymal membranes, and membrane fractions from undifferentiated or DMSO-treated U937 cells [6,16,24].

The N-terminal sequence and the second extracellular loop of the mCysLT$_1$R contain conserved putative glycosylation sites. These sites might play a role in the processing of the receptor as suggested by experiments treating THP-1 cells with tunicamycin, which resulted in a 6-fold decrease in the number of detectable specific binding sites [25]. Also, the third intracellular loop and C-terminus display several conserved phosphorylation sites. These sites could be critical in the uncoupling of the receptor to G protein or in the process of homologous/heterologous activation/desensitization of the CysLT$_1$ receptor [10,26,27].

The rank order affinity for the cysteinyl-leukotrienes, $LTD_4 \gg LTE_4 = LTC_4 \gg LTB_4$, is similar to that described for the human receptor [10]. This is consistent with previous experiments performed with DMSO-treated U937 cells and guinea pig lung membranes [16]. Thus, LTD_4 is the preferred ligand at this receptor, showing > 200-fold affinity compared with the other two cysteinyl-leukotrienes. This is in distinct contrast to the $CysLT_2R$ in which both LTD_4 and LTC_4 have similar binding affinities at the cloned receptors [12–14]. The rank order of binding affinities to the $mCysLT_1R$ of three structurally distinct classes of $CysLT_1R$ selective antagonists, MK-571, pranlukast (Onon™), and zafirlukast (Accolate™), is comparable to the recombinant expressed $hCysLT_1R$ [10,11] and to guinea pig lung membranes [28]. Although BAY-u9773 is described as a non-selective CysLT receptor antagonist in the competitive binding assay, it was 1000-fold less active compared with the selective $CysLT_1R$ antagonists. Recently, Nothacker *et al.* [14] described partial agonist properties of BAY-u9773 at the cloned human $CysLT_2R$.

Both expressed isoforms of $mCysLT_1$ receptor induce after activation an increase in Ca_i^{2+}. Activation of CysLT1 receptor in U937 cells [29], human epithelial cells [30], mesangial cells [31], and airway smooth muscle cells [32] also induces an increase in Ca_i^{2+}. These results are in agreement with results obtained with the $hCysLT_1R$ expressed in Cos-7 cells [10,11]. There is somewhat of a discrepancy in the ability of LTD_4 and LTC_4 to increase Ca_i^{2+} and to induce dispersion of melanosomes. This may be due to differences in cell type, affinities for G protein, or the particular G protein coupled to the receptor.

In summary, we have cloned and functionally characterized two potential mouse $CysLT_1R$ forms. Future studies with suitable antibodies will examine whether a single receptor or both forms are expressed in murine tissues at the protein level. These new molecular reagents will be important in gaining a more complete understanding of the pathological roles that the $CysLT_1R$ plays in asthma as well as in other allergic and inflammatory diseases.

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