Structural determinants regulating expression of the high affinity leukotriene B₄ receptor: Involvement of dileucine motifs and alpha helix VIII.

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Running title: Dileucine motifs in cell surface expression of BLT1

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SUMMARY

Mutational analysis of determinants located in the carboxyl terminal (C) tail of the high affinity leukotriene (LT) \( \text{B}_4 \) receptor, BLT1, was performed to assess their significance in BLT1 trafficking. When expressed in COS-7 cells, a BLT1 deletion mutant lacking the C-tail (G291stop) displayed higher numbers of binding sites and increased signal transduction compared to wild type (WT) BLT1. Addition of the C-tail from either the platelet-activating factor receptor or the LTD\(_4\) receptor, CysLT1, did not restore WT phenotype. Moreover, the number of \( \text{LTB}_4 \) binding sites was higher in the chimeras than in the WT BLT1, suggesting the requirement for specific structural determinants within the BLT1 C-tail. Elimination of a distal C-tail dileucine motif (L304-L305), but not the proximal (L292-L293) motif, altered BLT1 pharmacological characteristics and caused a moderate constitutive receptor activation. Surprisingly, all mutant receptors were efficiently delivered to the plasma membrane, but not to a greater extent than WT BLT1, as assessed by flow cytometry. Furthermore, substitution of L304-L305 prevented \( \text{LTB}_4 \)-induced BLT1 internalization. Molecular modeling of BLT1 on the bovine rhodopsin receptor scaffold strongly suggested the involvement of the distal dileucine motif (L304-305) in a hydrophobic core, including intra-helical interactions within \( \alpha\)-helix VIII and inter-helical interactions with residues of helix I. Disruption of this hydrophobic core is proposed to increase the population of receptors in the active form, to restrain their trafficking and to facilitate the activation of BLT1 as indicated by the increased maximal level of binding of the ligand and constitutive activation of the receptor.
INTRODUCTION

A variety of stimuli, including light, neurotransmitters, hormones and inflammatory lipid mediators produce their effects via activation of G-protein-coupled receptors (GPCRs). Taking into account the heterogeneity of ligands, it is interesting that all members of this superfamily of receptors share the same topology characterized by the presence of seven transmembrane α-helices. Conserved residues within subfamily A (related to rhodopsin) are localized throughout these helices, serving as reference points for sequence alignments. Biophysical and biochemical evidence indicate that upon photo-activation, relative movements of transmembrane (TM) 3 and TM6 occur in rhodopsin; similarly, rearrangement of the cytoplasmic proximal portion of TM7 relative to TM1 and of TM2 relative to the intracellular helix VIII were also observed (reviewed in 1). Through structural mimicry, despite the incredible diversity of ligands, GPCRs presumably share the core function of activation through similar conformational changes (2).

GPCRs exist in equilibrium between the inactive (R) and active (R*) conformations, as described in the cubic ternary model of GPCR activation (3). Stabilizing networks of intramolecular interactions constrain the receptor in an inactive conformation. Disruption of these stabilizing interactions leads to isomerization of the population of receptors into the active conformation (R*). Several mutations have been described to facilitate the isomerization of receptors from the R to the R* state, leading to higher basal receptor activity in the absence of agonist (1,4-6).

Regulation of activated receptors generally involves Ser/Thr phosphorylation by the GPCR specific kinases (GRKs) and binding of members of the arrestin family, the latter acting as adaptors between the receptor and components of the internalization machinery (7-10). Interestingly, arrestin-independent GPCR endocytosis has been reported (11). The adaptin protein (AP) complexes participate in the formation of clathrin-coated pits (CCP) and sorting of GPCRs to CCP (12). APs also bind specific sorting signal sequences in cytoplasmic domains of several transmembrane proteins such as the tyrosine-based motifs (NPxY and Yxxy, where x represents any amino acid and y is a residue with a bulky hydrophobic group) and dileucine-based sorting signals.
signals (10,13,14). The latter have been involved in different aspects of CCP trafficking of transmembrane proteins including GPCRs (13-22).

Extensive studies have defined the carboxyl-terminal (C) tail of GPCRs as a key domain for several aspects of the GPCR biology, including receptor activation and regulation. The atomic structure resolution of rhodopsin in its inactive form revealed the presence of an eighth helix, located in the C-tail segment distal to helix VII (23). Post-translational modifications such as phosphorylation and palmitoylation in the C-tail are involved in receptor coupling selectivity (24) and G-protein activation (25). Other motifs are recognized as docking sites for scaffolding proteins (26).

In the present study, we investigated whether specific determinants located in the C-tail were implicated in the activation and trafficking of the high-affinity human leukotriene (LT)B₄ receptor (BLT1). LTB₄ is a powerful inflammatory mediator derived from lipoxygenation of arachidonic acid which induces neutrophil chemotaxis, chemokinesis, aggregation, degranulation, cation fluxes, as well as other immunodulatory functions (reviewed in 27; 28-30). LTB₄ mediates its functions through interaction with two specific plasma membrane receptors showing different affinity for the ligand: a high-affinity LTB₄ receptor, BLT1 (31) and low affinity receptor, BLT2 (32), which share 45% identity. Several structural determinants purportedly involved in the regulation of transmembrane protein trafficking are present in the BLT1 C-tail and are conserved among species (human, rat, mouse, guinea pig) as well as in the human BLT2 (Fig. 1A).

We recently demonstrated that within the BLT1 C-tail reside structural elements involved in its desensitization as well as in arrestin-independent, GRK- and dynamin-dependent BLT1 internalization, both regulatory processes require an intact C-tail² (33). In addition, preliminary data revealed that a BLT1 deletion mutant (G291stop) lacking the C-tail, transiently expressed in COS-7 cells, showed higher numbers of LTB₄ binding sites and increased signal transduction when compared to the WT receptor (33). These data suggest that specific structural determinants of BLT1 C-tail regulate cell surface expression, yet the motifs involved are still unknown. Within this receptor segment are located most of the multiple potential intracellular phosphorylation sites
(Ser/Thr) as well as two dileucine motifs (Fig. 1A). We therefore sought to determine which structural determinants of BLT1 direct its expression.
EXPERIMENTAL PROCEDURES

Reagents — cDNA encoding $\alpha_{16}$ was a generous gift from Dr. M. I. Simon (Pasadena, CA, U.S.A.); cDNA encoding GRK2 was a generous gift from Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). Other materials and their sources were as follows: Geneticin and all culture media from Invitrogen Canada Inc. (Burlington, ON, Canada); bovine serum albumin (BSA), Hanks’ Balanced Salt Solution (HBSS), paraformaldehyde and poly-L-lysine from Sigma-Aldrich Canada Ltd. (Oakville, ON); fetal bovine serum (FBS) from BIO MEDIA Canada Inc. (Drummondville, QC); FuGENE-6 Transfection reagent, Pwo polymerase and restriction endonuclease from Roche (Mississauga, ON, Canada); T4 DNA ligase, anti-mouse antibody conjugated to horseradish peroxidase, $^{32}$P, $[^3H]$-LTB$_4$ and $[^3H]$-myo-inositol from Amersham Pharmacia Biotech Inc. (Baie D’Urfé, QC, Canada); LTB$_4$ from Cayman Chemical (Ann Arbor, MI); perchloric acid from VWR Canlab (Ville Mont-Royal, QC, Canada); fluorescein isothiocyanate-conjugated goat anti-mouse antibody from BIO/CAN Scientific (Mississauga, ON, Canada); gentamicin sulfate from Schering Canada Inc. (Pointe-Claire, QC).

Construction of Myc tagged wild type (WT) and mutant receptors — The cloning of WT BLT1 cDNA was previously described (34). In order to allow rapid assessment of cell surface expression of BLT1, the N-terminal initiator methionine was replaced by the Myc sequence MEQKLISEEDLSRGSPG resulting in Myc epitope-tagged WT and mutant BLT1. The G291stop mutant generation was also described previously (33). The chimeric mutant receptors were generated by PCR. Primers were designed to link BLT1 core receptor at residue G291 (Fig. 1A), immediately after the TM7, in-frame with the carboxyl-terminal tail of either PAFR C-tail, from residue Lys298, or CysLT1 C-tail, from residue Gly300. The 4L/A (in which L292, L293, L304 and L305 are substituted to alanine) or 2L(304-5)/A and 2L(292-3)/A mutant BLT1 were also constructed by PCR amplification. Each mutant BLT1 is identified by the single letter code of the original amino acid followed by the position number and the single letter code of the exchanged amino acid (Fig. 1B). All constructions were subcloned into pcDNA3 expression vector
(Invitrogen). Mutant and chimeric receptor cDNAs were then sequenced (University of Calgary, Alberta, Canada) to confirm substitutions or fusion of the chimeric receptor. The human Ga16 and GRK2 cDNAs were in pCIS, under the control of a CMV promoter, and in pcDNA3, respectively.

**Cell culture and transfections**—COS-7 and HEK 293 were grown in Dulbecco’s modified Eagle’s medium (DMEM) with glucose, supplemented with 10% FBS and gentamicin sulfate (40 μg/ml). Transient transfections were carried out with FuGENE-H™ and experiments were performed 48 h post-transfection. Total transfected cDNA quantities were adjusted, for each experiment, with the pcDNA3 vector DNA.

**Radioligand binding assay**—1.2 x 10⁶ COS-7 cells were transiently transfected with 2 μg of cDNA encoding WT or mutant BLT1. After 48 h, cells were harvested and washed twice in phosphate buffer saline (PBS) and twice in Hepes-Tyrode’s Buffer containing 0.1% (w/v) BSA (35) in which cells were also resuspended for the assay. Competition binding curves were carried out on 2 x 10⁵ cells with 0.25 nM [³H]-LTB₄ and increasing concentrations of non-radioactive LTB₄ for 2 h at 4°C. For saturation binding curves, 1 x 10⁵ cells were subjected to increasing [³H]-LTB₄ concentrations (0–16 nM). Non-specific binding was measured in presence of 2 μM of nonradioactive LTB₄ and represented less than 10% of total binding. Free radioactivity was separated from cells by centrifugation and a double wash with 1 ml Hepes-Tyrode’s buffer. Radioactivity contained in the cell pellet was counted in liquid scintillation using a β counter.

**Inositol phosphate determination**—COS-7 cells were plated in 30 mm dishes (2.0 x 10⁵ cells/dish) and cultured 24 h before transient transfection with cDNAs encoding WT or mutant BLT1 in combination with cDNA of Ga16 subunit. In brief, 24 h post-transfection, cells were labeled and the following day, stimulated with 100 nM LTB₄. Inositol phosphates (IP) were then extracted and radioactivity was counted as described previously (34). Total IP levels represent IP production in response to the agonist, over basal levels (or unstimulated). Constitutive activity was determined by comparing the ratio between the basal IP levels of mutant and WT receptors.

**Flow cytometry studies**—COS-7 cells transiently expressing the Myc-tagged WT or mutant BLT1 were subjected to flow cytometry analysis. Cells (2.5 x 10⁵) were labeled as
previously described (34) with anti-Myc followed by incubation with fluorescein isothiocyanate-
conjugated goat anti-mouse IgG antibody. All measures were performed on a FACSscan flow
cytometer using CellQuest software (Becton-Dickinson).

**Internalization assay** – HEK 293 grown at 80% confluence in petri dishes were
transfected with 2 μg of cDNA encoding WT or mutant BLT1 with 4 μg of each other cDNA or
the pcDNA3 expression vector DNA as indicated in figure legends. Transfected cells cultured for
24 h were transferred to 6-well plates with 1.5 x 10⁶ cells/well and incubated for another 24 h. After
washing once with HBSS, medium containing LTB₄ (300 nM) or its vehicle was added and the
cells were incubated for 1 h at 37 °C. Incubations were stopped on ice and cells were washed twice
with ice-cold HBSS. Flow cytometry analysis was carried out as described earlier except that
antibody labeling was performed at 4 °C while cells were still attached to the plates. Cells were
collected and analyzed. Percentage of receptor internalization was calculated based on either the
mean fluorescence intensity (MFI) value of cells or on the percentage of positive cells, with
comparable results, using LTB₄-treated cells compared to cells treated with the vehicle alone.

**Molecular modeling** – The modeling and rendering of BLT1 were executed using the
INSIGHTII suite of programs (Accel Ins., San Diego, CA). All the calculations were performed on
an SGI Octane2 workstation (Silicon Graphics Ins., Mountain View, CA.), as described previously
(36). Briefly, a pairwise sequence alignment between the primary structures of BLT1 (SwissProt
Accession number GenBank accession no. D89078) and the Bovine Rhodopsin (PDB: 1L9H) was
performed using the program HOMOLOGY. The sequences of the two proteins were aligned so as
to equivalence the positions of the following conserved residues: Asn⁵⁵-Asn¹.⁵⁰³⁶ (the
superscripts represent the residue numbering in rhodopsin structure PDB code 1F88, and human
BLT1 sequence, respectively, and ¹.⁵⁰ is the numbering in the standardized nomenclature), Asp³⁵-
Asp².⁵⁰⁴, Arg¹³⁵-Arg³.⁵⁰¹¹⁵, Trp¹⁶¹-Trp⁴.⁵⁰¹⁴², Pro²¹⁵-Pro⁵.⁵⁰¹⁹³, Pro²⁸⁷-Pro⁶.⁵⁰²³⁶ and Pro³⁰³-
Pro⁷.⁵⁰²³². The coordinates of the identified structurally conserved regions were then transferred
to the sequence of the BLT1. To relieve residual strain resulting from suboptimal positioning of the
side chains, the resulting model was subjected to energy minimization using the program
DISCOVER with consistent valence forcefield (37). During this process, the C\(_n\) atoms and the side chains of conserved residues were kept fixed at their positions in the rhodopsin crystal structure. A distance-dependent dielectric constant of 4 was used with simple harmonic potential for bond length energy. No cross-term energies were included and the peptide bonds were forced to planarity.

**Statistical analysis** – Data were analyzed for statistical significance using Student’s paired \(t\) test. Differences were considered significant at \(p<0.05\).
RESULTS

WT and mutant BLT1 were expressed in a mammalian expression system in order to determine and characterize structural elements involved in BLT1 trafficking and receptor activation. Motifs located in the carboxyl-terminal tail of the receptor were investigated with regard to receptor expression, targeting to the membrane and functionality.

Structural determinants of BLT1 C-tail are essential for regulation of BLT1 expression.

We previously reported that a complete C-tail-truncated BLT1 (G291stop) showed increased numbers of \(^{[3]H}\)-LTB\(_4\) binding sites and a greater inositol phosphates (IP) production in response to LTB\(_4\) stimulation than WT BLT1 (33). In order to define whether selective BLT1 determinants could regulate its cell surface trafficking, we created two chimeric receptors composed of the main core of BLT1: residues 1 to 291 at the end of TM7 of BLT1 and the C-tail of either the platelet-activated factor receptor (PAFR) or the LTD\(_4\) receptor CysLT1 (Fig. 1B). The amino acids present in the C-tail of each receptor are shown in Figure 1A. Chimeric receptors (BLT1-PAFR and BLT1-CysLT1) were transiently expressed in COS-7 cells and were present at the plasma membrane as assessed by \(^{[3]H}\)-LTB\(_4\) binding (Table I). Nonlinear regression analysis of competition binding curves revealed the presence of one class of binding sites exhibiting high affinity for LTB\(_4\) (BLT1-PAFR, \(K_d = 3.6 \pm 0.7\) nM; BLT1-CysLT1, \(K_d = 6.7 \pm 1.9\) nM). These \(K_d\) values were slightly higher than the value for WT BLT1 value (\(K_d = 1.0 \pm 0.5\) nM) (Table I).

We next investigated whether those chimeric receptors were able to transduce LTB\(_4\) signaling. Total IP accumulation was monitored in COS-7 cells coexpressing G\(\alpha_{16}\) subunit protein and either chimeric or WT receptor. Surprisingly, in response to 100 nM of LTB\(_4\), IP production was markedly increased for both BLT1-PAFR (155 \pm 16 %) and BLT1-CysLT1 (163 \pm 18 %) in comparison with WT (defined as 100 %) (Fig. 2A). Furthermore, the increased ability of the chimeric BLT1 to transduce LTB\(_4\) signaling was associated with a higher number of binding sites, as shown by \(^{[3]H}\)-LTB\(_4\) saturation binding curves (Fig. 2B). These results suggest that the C-tail of WT BLT1 contains a signal that is not conserved in PAFR or CysLT1 C-tail, which controls BLT1 trafficking or functional expression at the cell surface.
We further sought to define the structural determinants present in the C-tail that could regulate BLT1 expression. Since a partially C-tail-truncated BLT1 mutant (G319stop) had shown binding characteristics similar to WT BLT1 (33), we investigated the cytoplasmic segment comprising residues 291 to 319. Two types of structural determinants located within the 291-319 C-tail segment could be involved in receptor trafficking: phospho-acceptor sites (S/T) and dileucine motifs (LL). Notably, the addition of PAFR and CysLT1 C-tails containing potential phospho-acceptor sites did not prevent the increase in binding site numbers observed with the G291stop BLT1. Moreover, several phosphorylation sites (T308, S310, S313, S314, T315) within this segment had been mutated without any effect on [3H]-LTB4 binding (33; and data not shown). Consequently, BLT1 C-tail dileucine motifs were further studied.

Characterization of dileucine mutant BLT1.

Dihydrophobic motifs, such as leucine-valine and leucine-leucine are involved in efficient trafficking of several transmembrane proteins through sorting, localization or internalization signals as observed for the β2-AR and CXCR4 (13,20,21). Two dileucine motifs are located between residues 291-319 of the BLT1 C-tail. In order to assess whether the dileucines were essential for the WT phenotype, we used site-directed mutagenesis to disrupt L292, L293 and L304, L305 in the full-length BLT1. Leucines were substituted to alanines so as to change each motif alone or in combination, generating three new mutant receptors: 2L(292-3)/A, 2L(304-5)/A and 4L/A mutant BLT1 (Fig. 1B). Again, mutant receptors displayed a higher Kₐ for LTB₄ (respective Kₐ values: 4.47 ± 1.47 nM, 12.2 ± 3.0 nM, 10.6 ± 2.4 nM) than the WT BLT1 (Table I). LTB₄-induced IP accumulation was increased for the 4L/A mutant receptor (136 ± 17 %) when compared to the WT (defined as 100%) (Fig. 3A). Interestingly, mutation of the distal dileucine motif 2L(304-5)/A led to a significant increase in IP production upon LTB₄ exposure (177 ± 22 %), whereas mutation of the proximal motif 2L(292-3)/A had no detectable effect (108 ± 22 %). Similar results were also observed in a time course study of IP production (Fig. 3B).

We next addressed whether dileucines might regulate BLT1 expression. Saturation binding curves correlated with the IP production, given that the 4L/A and the 2L(304-5)/A mutant receptors...
showed higher numbers of binding sites at the plasma membrane, but saturation could not be achieved (Fig. 3C). In contrast, WT and the 2L(292-3)/A BLT1 binding sites for LTB₄ could be saturated. Overall, these results suggest that leucines 304 and 305 are responsible for the phenotype observed with the G291stop deletion.

We assessed the expression levels of Myc-tagged WT and mutant BLT1 at the plasma membrane by flow cytometry analysis. Surprisingly, the mean fluorescence intensity of COS-7 cells expressing each mutant receptor did not significantly vary from that of WT BLT1 (Fig. 4, Table II). Similar results were observed when the number of Myc⁺ cells was evaluated (Table II). Thus, the cell surface expression of the protein was not affected by removal of either one or both of the dileucine motifs and hence, the increased binding capacity of the population of receptors was not the consequence of a higher number of cell surface BLT1. Therefore, it could be hypothesized that the distal dileucine motif regulated in some way LTB₄ binding or LTB₄ mediated activation of BLT1, by an augmentation of the population of receptors in the active state.

When basal activity of the receptors was investigated in terms of IP accumulation in the absence of LTB₄, the 2L(304-5)/A and 4L/A mutant receptors showed an increase in constitutive activity, which represented approximately a 2-fold increase in basal IP production compared to the WT BLT1 (Fig. 5). In contrast, the basal IP production of the 2L(292-293)/A BLT1 did not significantly differ from the one observed with the WT BLT1. On the other hand, coupling selectivity of BLT1 mutant receptors did not change (33; and data not shown).

The distal dileucine motif is essential for BLT1 internalization.

Endocytosis of activated GPCRs is generally thought to involve receptor phosphorylation, mainly by GRKs, arrestin binding and internalization via CCP. We recently demonstrated² that BLT1 internalized in a dynamin- and clathrin-dependent manner in HEK 293 cells, but independently of arrestins. Since motifs like dileucines may contribute to receptor endocytosis, we evaluated the extent of BLT1 endocytosis by flow cytometry analysis of receptor loss at the plasma membrane in HEK 293 cells after stimulation with LTB₄. WT BLT1 endocytosis reached 27 %, in contrast to the 4L/A mutant receptor which did not internalize. Furthermore, the distal dileucine
motif was essential to agonist-induced endocytosis of BLT1 (2L(304-5)/A: -3 ± 4 %), whereas the proximal dileucine motif was not involved in this process (2L(292-3)/A: 31 ± 13 %) (Fig. 6, Table III).

**Molecular modeling reveals key hydrophobic interactions.**

We used molecular modeling to gain insight into the structure-function of the two dileucine motifs located within the C-tail of BLT1. We show in Figure 7A and B, a homology model of the BLT1. The bovine rhodopsin structure was used as template and homology modeling was performed as described in “Experimental Procedures”. As seen with the bovine rhodopsin structure, BLT1 shows a short amphipatic α-helix (helix VIII), which lies perpendicular to helix VII. It shows the two dileucine motifs located on the same face of this eighth helix, with the proximal L292-L293 motif at the beginning of the helix (in white) and the distal L304-L305 motif at the end of the helix (in yellow). It must be noted that this model represents the inactive form of the BLT1, since in the crystal structure of rhodopsin the receptor was in its inactive state. Analysis of the model indicates that L304-L305 are involved in a hydrophobic core with other hydrophobic residues in helix VIII (the highly conserved F300 and V301, in blue) and in helix I (F38, W41, L44, in green). This hydrophobic core could be involved in stabilizing the inactive state of the receptor.
DISCUSSION

Little is known about structural determinants regulating expression and trafficking of BLT1, the high affinity receptor for LTB₄. In this study, we show that a dileucine-based motif (L304-L305) located in the C-tail of BLT1 regulates both receptor internalization and the numbers of cell surface binding sites for LTB₄.

Cytoplasmic domains of GPCRs contain specific motifs involved in signal transduction and regulation, trafficking and receptor turnover. As a member of this superfamily of receptors, BLT1 contains several of these motifs that could serve as potential sorting signals: two tyrosine-based signals, YSD²⁰⁴ and NPVLY²⁸⁵, located respectively in the third intracellular loop and TM7, many phosphoacceptor residues (Ser/Thr) mostly located within the C-tail of the receptor and two C-tail dileucine-based sorting motifs (Fig. 1A).

Structural determinants responsible for BLT1 trafficking

A C-tail-truncated BLT1 (G291stop) demonstrated an increased number of binding sites for [³H]-LTB₄ as well as enhanced signal transduction in response to LTB₄ stimulation (33), whereas internalization was drastically reduced when compared to WT². The observed pharmacological properties could result from elimination of phosphorylation sites or dileucine motifs, whereas involvement of the tyrosine-based motifs seems unlikely.

Although the C-tail sequences of PAFR or CysLT1R contain several potential phosphorylation sites (Fig. 1A), their fusion to the G291stop did not allow recovery of the WT BLT1 binding and activation characteristics. Thus, it appears that structural determinants other than the potential phosphoacceptor sites are responsible for WT BLT1 binding and signal transduction properties. Furthermore, a partially C-tail-truncated receptor (G319stop) conserved the WT phenotype in terms of binding characteristics and IP production (33). Hence, we decided to focus on the region comprising residues 291-319. This segment contains a stretch of acidic residues and phosphoacceptor sites that could influence BLT1 trafficking. However, previous results (33) and our unpublished observations suggest that at least the phosphoacceptor sites T308, S310, S313, S314 and T315 are not responsible for the increased binding and activity of the truncated or
chimeric BLT1. We therefore aimed to determine whether the two dileucine motifs located in this region of the C-tail, L292-L293 and L304-L305, were involved in the phenotype observed with the G291stop BLT1.

Dileucine motifs, which are quite conserved among GPCRs, are involved in membrane protein internalization and localization into specific compartments of the cell (10,14-16). They are known as sorting signals to the lysosome (15) or to the basolateral surface (16) and have been implicated in trafficking and endocytosis of several transmembrane proteins (10,13,14). We discovered that disruption of the distal dileucine (L304-L305) motif resulted in a mutant BLT1 with a higher number of binding sites on the cell surface, similar to what was observed for the C-tail-truncated G291stop and for the BLT1 chimeras. In contrast, the 2L(292-3)/A mutant BLT1 showed only a slight increase in the number of binding sites. The enhanced signal transduction observed with the 2L(304-5)/A and 4L/A mutants could be ascribed to a higher number of receptors on the cell surface. Indeed, Schulein and collaborators showed that a dileucine sequence and an upstream glutamate residue in the C-tail of the vasopressin V2 receptor was essential for cell surface delivery (38). However, BLT1 in which one or both dileucine-based motifs were disrupted were equally expressed at the plasma membrane, in spite of different numbers of binding sites, suggesting that the dileucine motif may have different roles in selected GPCRs. Interestingly, the dileucine-based hydrophobic motifs are conserved in both BLT receptors, as seen in the BLT2 primary structure.

**BLT1 internalization**

It has been proposed that sorting of proteins during endocytosis and biosynthesis is achieved via similar mechanisms (39). Clathrin-coated pit formation recruits a clathrin lattice and the AP-2 complex to the plasma membrane, whereas AP-1 is lured to the trans-Golgi network. In this process, dileucine motifs interact with the β1 subunit of AP-1 (40), directing the sorting of proteins to the trans-Golgi network. The structural determinants and the mechanisms underlying BLT1 endocytosis are still not defined, but we have previously demonstrated that BLT1 C-tail is essential for BLT1 internalization, as it is for a vast number of GPCRs, and that this phenomenon is uniquely
independent of arrestins. Herein, we show that changing the C-tail L304-L305 into alanines within the full-length BLT1 inhibits LTB₄-induced receptor endocytosis in HEK 293 cells.

**L292-L293 are not involved in BLT1 regulation**

Surprisingly, the proximal dileucine motif (L292-L293) substitution caused only a slight increase in the number of binding sites. In Lamp1, relative spacing of the motif YxxΦ from the seventh TM was shown to be important (41). Type II integral membrane proteins expose an acidic residue membrane-distal relative to a dileucine motif, whereas in membrane type I proteins, the acidic residue is membrane-proximal relative to the dileucine. These sequences have been proposed to exist in two forms: membrane-distal accessible dileucines mediate direct transport from the trans-Golgi network to endosomes, whereas membrane-proximal non-accessible dileucines are involved in the regulation of trafficking from the plasma membrane to endosomes. The latter motifs have been suggested to become accessible through phosphorylation of an adjacent Ser/Thr residue (19), although such phosphoserine-dependent AP1 binding has also been shown for the membrane-distal dileucine motif in the mannose-6-phosphate receptor (42). The proximal dileucine motif in BLT1 is located immediately after three glycines, which seem to delimit the end of the seventh TM. Therefore this motif might be positioned too close to the seventh TM to be recognized. Some dileucine-based endocytic signals are recognized constitutively or upon post-translational modification such as phosphorylation of a nearby amino-terminally positioned phosphoacceptor residue (21,43-45), which may open the structure exposing the signal sequence or simply stabilizing the interaction with the endocytic apparatus.

**BLT1 activation**

The distal dileucine motif L304-L305 of BLT1 C-tail thus appears to regulate the population of receptors in the active state and, hence, the number of binding sites for LTB₄. More binding sites are detected when the C-tail is removed or the distal dileucine motif is abrogated, suggesting that WT BLT1 binding sites may in fact represent only a fraction of the total LTB₄ binding capacity of the cell. In order to obtain structural information on the distal dileucine motif
and possibly a functional explanation for the characteristics displayed by mutant BLT1, we used molecular modeling.

Modelization of BLT1 structure on the bovine rhodopsin model.

The homology model of the BLT1 was based on the crystal structure of bovine rhodopsin and was obtained as described elsewhere (36). In our model, it can be seen that the two dileucine motifs are located within helix VIII and that the distal motif is involved in an hydrophobic core containing other hydrophobic residues in helix VIII (of which the strictly conserved Phe$^{313}$-Phe$^{7.60}$ and in helix I. A similar hydrophobic core stabilizes ICL4 of bovine rhodopsin in addition to the palmitoylated cysteines that anchor the helix to the membrane. Thus, this structural feature could have a functional role in stabilizing the inactive state of the receptor.

It has been reported that constitutive activation confers a higher degree of conformational flexibility to the β$_2$-adrenergic receptor due to the disruption of stabilizing conformational constraints. This higher degree of conformational flexibility may allow the constitutively active mutant to more readily undergo transitions between the lower energy, or inactive state and the higher energy, or active state (46). Mutation of the distal leucine side-chains to less hydrophobic alanine side-chains can be envisioned in the context of the present model to destabilize this inactive state of the receptor. With the proviso that the L304-305/A mutation destabilizes the inactive state and that we can neglect the effect of the mutation on the active state, it can be hypothesized that the mutant BLT1 will be more easily activated or will have a smaller free energy ($\Delta G$) of activation than the WT receptor (Fig. 7C).

One way to experimentally verify this hypothesis, i.e. that the $\Delta G$ of activation is smaller or that the population of receptor in the active state is greater, is to assess the activity of the receptor in absence of agonist or to detect increased constitutive activity for the mutant BLT1. Indeed, we found the existence of such increased constitutive activity for the 2L(L304-5)/A mutant receptor, therefore suggesting that this mutant has a decreased $\Delta G$ of activation and that the receptor is destabilized in its inactivated form. Of course, the active state of the receptor could also be stabilized by the
2L(304-5)/A mutation, but, unfortunately, such a possibility cannot be modeled at present since no direct structural information exists about the conformational changes in the active form of the receptor. On the other hand, additional evidence from thermodynamic models of receptor occupancy can be used to support the fact that the mutant 2L(304-5)/A BLT1 is activated more readily than the WT receptor in presence of the same concentration of agonist, leading to a greater population of receptors involved in signal transduction (47). Moreover, G-protein interaction is suggested to stabilize the active state of GPCRs and has been shown to promote higher affinity of a receptor for its ligand (48). Experimentally, this results in mutant BLT1 showing moderate constitutive activity and a higher number of LTB₄-binding sites.

On the other hand, there is evidence suggesting that dimerization can modify significantly the pharmacological profile of GPCRs, including BLT1 (48,49). However, the C-tail domain is not responsible for the dimerized state of BLT1 (48). As an emerging concept, the involvement of signaling complexes (50), formed even before agonist exposure, presupposes the existence of interactions between receptors and intracellular proteins associated with the signalitic machinery or with scaffolding. Such BLT1 interactions with intracellular proteins could modulate the numbers of LTB₄ binding sites made available by cell surface receptors. This interaction would be dependent on an intact distal dileucine-based motif. In a slightly different angle, interaction with intracellular proteins may keep BLT1 in signalitic complexes, such as raft microdomains, and therefore regulate LTB₄ binding. One could propose that, when receptor conformation is affected by mutation of the distal dileucine motif, the interactions mentioned above are impaired.

Curiously, removal or switching of the BLT1 C-tail with PAFR or CysLT1 did not modify the coupling selectivity of BLT1 (33; and data not shown). This latter observation suggests that helix VIII is not interacting directly with the G-protein. It is plausible that helix VIII may hide high affinity sites for the G-protein and that, upon activation of the receptor, the movement of this amphipathic helix reveals those high affinity sites, mainly located in the cytoplasmic loops. Moreover, crosslinking data from bovine rhodopsin suggest that the conformational movement of helix VIII upon activation is quite large (51,52).
During the final preparation of this manuscript, Okuno and collaborators published *on-line* an elegant demonstration that the helix VIII and the distal dileucine motif of BLT1 may be involved in the conformational changes required for receptor desensitization following G-protein activation (53). As extensively shown, GRKs are involved in both GPCR desensitization and internalization, even if these processes can be fully distinguished from one another. The presence of T308 near helix VIII is interesting since it is targeted by GRK6 in BLT1 desensitization following long-term exposure to LTB₄ (33). We cannot exclude that phosphorylation of T308 by GRKs might also be a relevant event in BLT1 internalization, opening the C-tail structure and allowing accessibility of the L304-L305 motif. On the other hand, neither substitution of T308 for alanine nor deletion of the BLT1 C-tail from residue 306 changed the receptor pharmacological characteristics compared to WT BLT1, in terms of ligand binding properties and basal receptor activity (33, 53). The latter observations may be associated with the fact that residue T308 is located outside of helix VIII.

**Conclusion**

With the present investigation, we provide evidence that a specific dileucine motif in a GPCR helix VIII is actively involved in modulation of ligand binding through regulation of the receptor activation process. In addition, we demonstrate for the first time that agonist-induced internalization of a GPCR depends on the helix VIII dileucine-based motif. Since BLT1 internalizes through a dynamin- and clathrin-dependent, but arrestin-independent pathway, the search for adaptor proteins that promote BLT1 sequestration remains open.
REFERENCES


Gaudreau et al.                                                                 Regulation of cell surface expression of BLT1.


FOOTNOTES

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1 Abbreviations: BLT1, leukotriene B₄ receptor; BSA, bovine serum albumin; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; G-protein, GTP-binding regulatory protein; GPCR, G-protein-coupled receptor; GRK, GPCR kinase; HBSS, Hanks' Balanced Salt Solution; IP, inositol phosphate(s); LTB₄, leukotriene B₄, PBS, phosphate buffer saline; PI, phosphoinositide; RT, room temperature; TM, transmembrane; TBS, Tris-buffered saline; WT, wild type.


Key words: Dileucine, internalization, expression, trafficking, BLT1, binding.
FIGURE LEGEND

Fig. 1. Schematic representation of BLT1, BLT2, PAFR and CysLT1 C-tail amino acid sequences, as well as chimeric receptors and point mutations of BLT1.  
A. The human BLT1, BLT2, PAFR and CysLT1 sequences are shown from the conserved NPxxY motif located at the end of TM7 of each receptor. Sequences were aligned with Dialign software. Asterisks point the dileucine motifs. B. Chimera and point mutant BLT1 sequences are shown. The single letter code is used, and the leucines (L) targeted by site-directed mutagenesis are shown in boldface. PAFR and CysLT1 C-tail sequences in the chimeric receptors are underlined.

Fig. 2. C-tail truncated and chimerics mutant BLT1 showed increased inositol phosphate production and LTB₄-binding sites. COS-7 cells were transiently transfected with the Myc epitope-tagged WT or mutant (BLT1-PAFR or BLT1-CysLT1) receptor cDNAs. A. IP responses were measured in cells also overexpressing Gα₁₆ subunit. Cells were exposed to 100 nM LTB₄ for 30 min at 37 °C and the total IP accumulation was determined, as described under “Experimental Procedures”. Each value represents the mean ± S. E. M of four independent experiments. B. Saturation binding curves were performed with increasing [³H]-LTB₄ concentrations (0-16 nM). A representative experiment of two is shown. ** p<0.01 versus WT BLT1-transfected cells.

Fig. 3. Dileucine mutant receptors showed increased inositol phosphate production and LTB₄-binding sites. COS-7 cells were transiently transfected with the Myc-tagged WT or mutant (4L/A, 2L(304-5)/A, 2L(292-3)/A) receptor cDNAs. A. IP responses were measured in cells also overexpressing Gα₁₆ subunit. Cells were exposed to 100 nM LTB₄ for 30 min at 37 °C and the total IP accumulation was determined, as described under “Experimental Procedures”. Each value represents the mean ± S. E. M of four independent experiments done in duplicate. B. A time-course study of IP accumulation, representative of three experiments, is shown. Cells expressing WT BLT1 stimulated for 30 min with 100 nM LTB₄ are defined as 100%. C. Saturation binding
curves were performed with increasing concentration of [³H]-LTB₄ (0-16 nM). A representative experiment of two is shown. * p<0.05, ** p<0.01 versus WT BLT1-transfected cells.

Fig. 4. Exchange of BLT1 C-tail with PAFR or CysLT1 or mutation of BLT1 dileucine-based motifs did not modify cell surface expression of receptors. COS-7 cells transiently expressing Myc-tagged WT or mutant BLT1 were assessed for cell surface expression by flow cytometric analysis. 48 h post transfection cells were first labeled with anti-myc antibody, followed by FITC, as described under “Experimental Procedures”. Each value represents the mean ± S. E. M of four independent experiments.

Fig. 5. Basal activity of WT and dileucine-mutant BLT1. Basal receptor activity was evaluated with COS-7 cells transiently overexpressing the Myc-tagged WT or mutant (4L/A, 2L(304-5)/A, 2L(292-3)/A) receptors and Gα₁₆ subunit. Basal IP accumulation was measured over 30 min at 37 °C in absence of agonist as described under “Experimental Procedures”. Each value represents the fold increased over WT basal level. The mean ± S. E. M of four independent experiments done in duplicate is illustrated. ** p<0.01 versus WT BLT1-transfected cells.

Fig. 6. Effect of disruption of C-tail dileucine-based motifs on BLT1 internalization. Myc-tagged WT and mutant BLT1 were transiently expressed in HEK 293 cells. 48 h post-transfection, cells were exposed or not to 300 nM LTB₄ and expression was assessed by cytofluorometric analysis. Receptors were labeled with anti-Myc antibody followed by FITC labeling as described in “Experimental Procedures”. Percentage of receptor loss from cell surface was determined from the mean fluorescence intensity (MFI) of cells. The results are the means ± S. E. M of four independent experiments, each done in duplicate.

Fig. 7. Molecular model of BLT1 based on bovine rhodopsin crystal structure. A and B. A molecular model of BLT1 structure was generated based on the bovine rhodopsin molecular model
as described in “Experimental Procedures”. The seven transmembrane helices are shown with the following color coding: helix I, green; II, purple; III, red; IV, kaki; V blue; VI, brown; VII, white; is also shown in blue helix 8 which is perpendicular to helix VII. Spaced filling representation of residues L292-L293 (white), L304-L305 (yellow) and those forming intra-helical (blue) and inter-helical (green) contacts with L304-L305. C. Simplified thermodynamic energy state diagram for activation of GPCR. At equilibrium, the population of receptors in each state is determined by its relative energy and the population of receptors in the active form is determined by the ability of the receptors to overcome the energy difference between the R and R* states.
Table I

Binding characteristics of WT and modified BLT1.

WT and mutant BLT1 were transiently expressed in COS-7 cells and assessed for ligand binding as described in “Experimental Procedures”. Dissociation constants (Kₐ) were calculated from competition binding curves, using GraphPad Prism version 3.0a (GraphPad Software, San Diego California USA, www.graphpad.com). The results are the means ± S.E.M of three independent experiments, each done in duplicate.

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Dissociation constant, Kₐ (nM)</th>
</tr>
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<tbody>
<tr>
<td>BLT1 WT</td>
<td>0.99 ± 0.46</td>
</tr>
<tr>
<td>BLT1-PAFR</td>
<td>3.59 ± 0.74</td>
</tr>
<tr>
<td>BLT1-CysLT1</td>
<td>6.71 ± 1.87</td>
</tr>
<tr>
<td>4L/A</td>
<td>10.6 ± 2.40</td>
</tr>
<tr>
<td>2L(304-5)/A</td>
<td>12.2 ± 3.00</td>
</tr>
<tr>
<td>2L(292-3)/A</td>
<td>4.47 ± 1.47</td>
</tr>
</tbody>
</table>
Table II

Expression of WT and mutant BLT1.

Cell surface expression of Myc-tagged WT and mutant BLT1 expressing COS-7 cells was assessed by cytofluorometric analysis 48 h post-transfection. Receptors were labeled with anti-Myc antibody followed by FITC labeling as described in “Experimental Procedures”. Percentage of the positive cell population and the mean fluorescence intensity (MFI) of cells are reported. The results are the means ± S.E.M of at least three independent experiments, each done in duplicate.

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Positive cells (%)</th>
<th>MFI (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>48.7 ± 2.9</td>
<td>61.1 ± 6.1</td>
</tr>
<tr>
<td>G291stop</td>
<td>37.7 ± 0.7</td>
<td>38.2 ± 5.3</td>
</tr>
<tr>
<td>BLT1-PAFR</td>
<td>40.4 ± 2.7</td>
<td>43.7 ± 7.4</td>
</tr>
<tr>
<td>BLT1-CysLT1</td>
<td>35.2 ± 1.7</td>
<td>49.2 ± 13.2</td>
</tr>
<tr>
<td>4L/A</td>
<td>43.7 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>2L(304-5)/A</td>
<td>51.0 ± 4.1</td>
<td>51.4 ± 5.2</td>
</tr>
<tr>
<td>2L(292-3)/A</td>
<td>42.3 ± 2.8</td>
<td>59.9 ± 12.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>61.9 ± 9.2</td>
</tr>
</tbody>
</table>
Table III

Internalization levels of WT and mutant BLT1.

Myc-tagged WT and mutant BLT1 were transiently expressed in HEK 293 cells. 48 h post-transfection, cells were exposed to 300 nM LTB₄ or its vehicle (ethanol) and expression was assessed by cytofluorometric analysis. Receptors were labeled with anti-Myc antibody followed by FITC labeling as described in “Experimental Procedures”. Percentage of receptor loss from cell surface was determined from the Myc⁺ cell population and the mean fluorescence intensity (MFI) of cells. The results are the means ± S.E.M of four independent experiments, each done in duplicate.

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Positive cells (%)</th>
<th>MFI (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>15.4 ± 5.8</td>
<td>26.6 ± 5.8</td>
</tr>
<tr>
<td>4L/A</td>
<td>0.2 ± 4.6</td>
<td>-13.3 ± 8.2</td>
</tr>
<tr>
<td>2L(304-5)/A</td>
<td>1.9 ± 3.3</td>
<td>-3.0 ± 3.9</td>
</tr>
<tr>
<td>2L(292-3)/A</td>
<td>8.5 ± 7.8</td>
<td>31.5 ± 13.5</td>
</tr>
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Figure 1
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