Identification of the Intracellular Region of the Leukotriene
B4 Receptor Type 1 That Is Specifically Involved
in G, Activation*

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Many G-protein-coupled receptors can activate more than one G-protein subfamily member. Leukotriene B4 receptor type
1 (BLT1) is a high affinity G-protein-coupled receptors for leukotriene B4 functioning in host defense, inflammation, and
immunity. Previous studies have shown that BLT1 utilizes different G-proteins (the Gg family and G16 G-proteins) in mediating
diverse cellular events and that truncation of the cytoplasmic tail of BLT1 does not impair activation of Gg and G16
proteins. To determine responsive regions of BLT1 for G-protein coupling, we performed an extensive mutagenesis study of
its intracellular loops. Three intracellular loops (I1, I2, and I3) of BLT1 were found to be important for both Gg and G16 coupling,
as judged by Gg-dependent guanosine 5'-[y-thio]triphosphate (GTPyS) binding and G16-dependent inositol phosphate accumulation assays. The i3-1 mutant, with a mutation at the i3 amino terminus, exhibited greatly reduced GTPyS binding but intact inositol phosphate accumulation triggered by leukotriene B4 stimulation. These results suggest that the i3-1 region is required only for Gg activation. Moreover, in the i3-1 mutant, the deficiency in Gg activation was accompanied by a loss of the high affinity leukotriene B4 binding state seen with the wild type receptor. A three-dimensional model of BLT1 constructed based on the structure of bovine rhodopsin suggests that the i3-1 region may consist of the cytoplasmic end of the transmembrane helix V, which protrudes the helix into the cytoplasm. From mutational studies and three-dimensional modeling, we propose that the extended cytoplasmic helix connected to the transmembrane helix V of BLT1 might be a key region for selective activation of Gg proteins.

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Leukotriene B4 (LTB4) is an inflammatory lipid mediator biosynthesized from membrane phospholipids by sequential
enzymatic reactions (1–4). LTB4 recruits and activates leukocytes, such as granulocytes, macrophages, monocytes, and
T-cells, and plays pivotal roles in host defense and the pathogenesis of inflammatory and immune diseases (5, 6). These
actions are mediated by two types of G-protein-coupled receptors (GPCRs). One is the LTB4 receptor type-1 (BLT1), a high
affinity LTB4 receptor highly expressed in leukocytes (7). The other, BLT2, is a low affinity LTB4 receptor expressed more
ubiquitously than BLT1 in human tissues (8–11).

Many GPCRs can activate more than one G-protein subfamily member. For example, the prostaglandin E2 receptor EP3D
 couples to Gg and G12 (12), platelet-activating factor receptor activates Gg and G12 (13, 14), and the sphingosine 1-phosphate
receptors S1P1 and S1P2 activate Gg and G12/13 (15). Previous reports suggest that in mediating different cellular events,
BLT1 also utilizes different G-proteins, namely pertussis toxin (PTX)-sensitive Gg and PTX-resistant G16 subfamilies of
G-proteins (7, 16). Promiscuous G-protein coupling enables GPCRs to mediate diverse signals.

The molecular determinants of many receptors participating in G-protein coupling and activation have been investigated.
With some exceptions, most of these studies emphasize the role of membrane-proximal regions of the second and third
intracellular loops and of the cytoplasmic tail of the receptor (17). However, no consensus sequences for G-protein coupling have been

We and others have reported that truncation of its cytoplasmic
tail does not impair the ability of BLT1 to activate G-proteins (18, 19). To identify regions of BLT1 responsible for
G-protein coupling, we performed an extensive mutagenesis study of its intracellular domains. Here, we show that BLT1
distinguishes Gg from G16 at the amino terminus of the third
intracellular region (the i3-1 region). Furthermore, Scatchard analyses of the i3-1 mutant revealed that BLT1 requires Gg
coupling to maintain a high affinity state for LTB4. A three-dimen-

The abbreviations used are: LTB4, leukotriene B4; GPCR, G-protein-coupled receptor; BLT1, leukotriene B4 receptor type 1; GTPyS, guanosine 5'-[y-thio]triphosphate; IP, inositol phosphate; HA, hemagglutinin; HEK293, human embryonic kidney 293 cell line; CHO, Chinese hamster ovary cell line; BSA, bovine serum albumin; PTX, pertussis toxin; WT, wild type.
sional model of BLT1 suggested that the i3-1 region might localize to the cytoplasmic end of the transmembrane helix V. This region is suggested to form an extended helical structure proximate to the membrane, which projects toward the cytoplasm in order to interact with G\(_i\).

**EXPERIMENTAL PROCEDURES**

**Materials**—cDNAs encoding Go11, Go12, Go13, Go14, Go14, and Go\(\alpha_6\) were obtained from the Guthrie cDNA Resource Center. LT\(_B_1\) was purchased from Cayman Chemical (Ann Arbor, MI). \(^3\text{H}\)LT\(_B_4\) and \(^35\text{S}\)GTP\(_\gamma\)S were from PerkinElmer Life Sciences. myo-\(^3\text{H}\)Inositol was from Amersham Biosciences. Anti-hemagglutinin (HA) antibody (clone 3F10) was from Roche Applied Science (Penzberg, Germany), and goat anti-rat IgG was from G\(\alpha_6\) horseradish peroxidase was from Santa Cruz Biotechnology, goat anti-rat IgG-sham Biosciences. Anti-hemagglutinin (HA) antibody (clone 3F10; 1:25,000) was from Covance (Ann Arbor, MI). \(^3\text{H}\)LTB4 and \(^35\text{S}\)GTP\(_\gamma\)S were from PerkinElmer Life Sciences.

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**Chemical Binding Assay**—CHO stable cells were plated at 3.2 \times 10^4 cells/well in a 96-well plate. On the following day, cells were washed with buffer A (Hanks’ balanced salt solution; Invitrogen) containing 0.1% BSA), pretreated with buffer A containing 0.5 \mu M.
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3-isobutyl-1-methylxanthine for 15 min at room temperature, and then stimulated with a ligand solution (Buffer A, 0.5 μM 3-isobutyl-1-methylxanthine and 50 μM forskolin) containing various concentrations of LT_{B4} for 30 min at room temperature. The reaction was terminated by the addition of lysis buffer (Hanks’ buffered salt solution containing 1% Tween 20). cAMP concentrations in the lysate were determined by an Alpha-Screen cAMP assay kit (PerkinElmer Life Sciences).

Inositol Phosphate (IP) Accumulation Assay—Cells were seeded at 1 × 10^5 cells/well in a 12-well plate and transfected with WT BLT1 and/or G-protein α subunit on the following day. Twenty-four hours later, culture medium was replaced with fresh medium containing 1 μCi/ml myo-[^3H]inositol (Amer sham Biosciences), followed by incubation for another 18–24 h. Cells were washed twice and preincubated for 10 min in Dulbecco’s modified Eagle’s medium containing 0.1% BSA and 10 mM LiCl, followed by stimulation with various concentrations of LT_{B4} or vehicle for 30 min. Stimulation was terminated by replacing the medium with ice-cold 5% HClO_4 and incubating on ice for 2 h. Accumulated IP was purified by ion exchange chromatography (AG1-X8 resin; Bio-Rad), and radioactivity was measured by scintillation counting (20, 21).

Chemotaxis Assay—Polycarbonate filters with 8-μm pores (Neuro Probe Inc., Gaithersburg, MD) were coated with 10 μg/ml fibronectin in phosphate-buffered saline for 60 min. The filter was placed in a 96-well Boyden chamber (Neuro Probe) containing LT_{B4} solution in the lower wells. CHO cells stably expressing WT or mutated BLT1 were placed at 6 × 10^4 cells/well in the upper wells. After incubating at 37 °C in 5% CO_2 for 4 h, cells on the filter were fixed with methanol and stained using a Diff-Quik staining kit (International Reagents Corp., Kobe, Japan). The upper side of the filter was scraped free of cells. Then the number of cells migrating out to the lower side was determined by measuring optical densities at 595 nm using a 96-well microplate reader (Bio-Rad).

Statistical Analysis—Data were analyzed for statistical significance using analysis of variance using Prism 4 software (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at p < 0.05, 0.01, or 0.001, as indicated.

RESULTS

Mutagenesis—To determine regions of human BLT1 responsible for G_i and G_q protein activation, we performed extensive mutagenesis focusing on the intracellular loops. Three to four tandem amino acids in three regions (i1, i2, and i3) were replaced with alanines by PCR using HA-tagged human BLT1 as a template. We initially constructed 13 mutants designated i1-1 to i1-3, i2-1 to i2-5, and i3-1 to i3-5 (Fig. 1, A and B). Expression levels of these receptors on the cell surface were semiquantitatively analyzed by flow cytometry as follows. Cells transfected with WT BLT1 or mutants were stained with anti-HA antibody specific to the amino-terminal HA tag and Alexa-Fluor 488-conjugated secondary antibody. WT BLT1 and mutant receptors were expressed on the cell surface, judging from the increased means of fluorescence intensity of the WT BLT1 and mutants compared with the mock transfec tant (Fig. 1C).

BLT1/Gα Coupling—To determine which G_i protein subtypes couple to BLT1, we examined LT_{B4}-induced GDP-GTP exchange of Gα by a GTPγS binding assay using membrane fractions of HEK293 cells transiently expressing BLT1 and various G-protein α subunits. Transfection of BLT1 without G-proteins caused only a slight increase above basal levels in GTPγS binding upon application of LT_{B4} (Fig. 2A). When BLT1 was co-transfected with members of the G_i family of G-protein α subunits, GTPγS binding activities were enhanced, whereas co-transfection of Gα_{12} did not show such an effect (data not shown). Co-transfected Gα_{12} showed a significant increase in LT_{B4}-induced GTPγS binding. Moreover, LT_{B4}-induced GTPγS binding was completely abolished by pretreating the cells with PTX prior to membrane preparation (see Fig. 4A). These results suggest that measuring LT_{B4}-induced GTPγS binding is an appropriate way to evaluate activation of PTX-sensitive G_i proteins by BLT1.

The alanine mutants described above were expressed in HEK293 cells along with Gα_{12} and evaluated for GDP-GTP exchange of Gα by the GTPγS binding assay. All mutants showed lower GTPγS binding than WT, but the i1-3, i2-4, i3-1, and i3-2 mutants exhibited significantly reduced GTPγS binding (Fig. 2B). These results indicate that all three regions, i1, i2, and i3, are crucial for G_i coupling.

Next, to determine whether activation of the G_q family of G-proteins by mutant receptors is altered, we examined LT_{B4}-induced total IP accumulation in COS-7 cells expressing both BLT1 and various Gα subunits. As reported by Gaudreau et al. (20), COS-7 cells expressing both BLT1 and Gα_{q16} show significantly higher IP accumulation than cells expressing BLT1 alone (Fig. 3A). BLT1 mutants were co-expressed in COS-7 cells with Gα_{q16} and tested for their ability to activate Gα_{q16} by the IP accumulation assay. Among the mutants that showed impaired GTPγS incorporation, only the i3-1 mutant displayed normal IP accumulation after ligand application (Fig. 3B). The i3-4 and i3-5 mutants exhibited higher IP accumulation than WT BLT1.

Since these results suggested that the i3-1 region is responsible for G_q activation, we constructed another mutant in the more membrane-proximal region of the i3 loop (i3-0; Fig. 1B). As with the i3-1 mutant, the i3-0 mutant BLT1 showed normal IP accumulation but significantly reduced GTPγS binding after LT_{B4} application (Figs. 2B and 3B). These results show that the amino terminus of the i3 loop is important for G_q activation.
Effects of the i3-1 Mutation on $G_{i2}$ and $G_{16}$ Coupling—LTB$_4$ stimulated an increase in GTP$\gamma$S binding in a dose-dependent manner in HEK293 cells transfected with WT BLT1 and $G_{i2}$, but no significant increases were seen with the i3-1 mutant (Fig. 4A). Increases in GTP$\gamma$S binding mediated by LTB$_4$ completely disappeared following pretreatment of cells with 100 ng/ml PTX. These results suggest that the i3-1 region functions in $G_{i2}$ activation. However, in LTB$_4$-induced IP production, dose-response curves were similar between WT BLT1 and the i3-1 mutant in the presence of $G_{16}$ in COS-7 cells, and PTX pretreatment did not affect IP accumulation (Fig. 4B). These results further support the idea that the i3-1 region of BLT1 leads to impairment in PTX-sensitive $G_{i2}$ activation without affecting PTX-resistant $G_{16}$ signaling.

To gain further insight into the role of the i3-1 region in signal transduction, we established polyclonal CHO cells stably...
expressing WT BLT1 or the i3-1 mutant. Cells highly expressing WT or the i3-1 mutant receptor were collected by cell sorting. The expression level of i3-1 mutant was similar to that of WT BLT1, as judged from flow cytometric and Western blotting analyses (Fig. 5, A and B). In CHO stable cells, the maximum response of GTPγS binding with the i3-1 mutant was only one quarter (23%) that of WT BLT1, although WT and the i3-1 mutant showed LTB₄-induced GTPγS binding in a dose-dependent manner (Fig. 5C). The dose-response curve of LTB₄ with the i3-1 mutant was shifted to the right from that seen with WT BLT1 (EC₅₀ 34 versus 5.0 nM) (Fig. 5, C and D). EC₅₀ values of LTB₄-induced adenyl cyclase inhibition in cells expressing the i3-1 mutant and WT BLT1 receptors were 6.0 and 0.25 nM, respectively (Fig. 5E). It has been reported that GPCR-dependent chemotaxis requires activation of Gᵢ and a resulting release of Gβγ dimers (26) and that LTB₄-induced chemotaxis is completely PTX-sensitive (7). Although both WT and the i3-1 mutant BLT1 cells showed chemotactic responses to LTB₄ with bell-shaped dose-response curves, cells expressing the i3-1 mutant showed impaired migration to low concentrations of LTB₄ (Fig. 5F). However, IP accumulation in WT and the i3-1 mutant BLT1 cells transfected with Gᵢ₁₆ were comparable (Fig. 5G). These results confirm the presence of both impaired Gᵢ activation and normal Gᵢ₁₆ activation by the i3-1 mutant in the same CHO stable cells.

Effects of the i3-1 Mutation on [³H]LTB₄ Binding—To examine the effect of the i3-1 mutation on ligand binding, we performed [³H]LTB₄ binding experiments. Membrane preparations (10 μg of protein) from HEK293 cells transiently expressing WT BLT1 or the i3-1 mutant were incubated with various concentrations of [³H]LTB₄ (Fig. 6A). From Scatchard analyses, WT BLT1 showed high affinity LTB₄ binding with a Kᵣ value of 3.5 nM and Bₘₐₓ value of 2.1 pmol/mg protein (Fig. 6B). In contrast, the i3-1 mutant showed low affinity LTB₄ binding with a Kᵣ of 6.4 nM and a Bₘₐₓ value of 1.8 pmol/mg protein. To analyze whether Gᵢᵩ coupling is required for high affinity LTB₄ binding, we examined LTB₄ binding of membrane preparations of PTX-treated cells. WT BLT1-expressing cells pretreated with PTX displayed only low affinity LTB₄ binding similar to the i3-1 mutant (Fig. 6B).

Experiments Using Monoclonal Cell Lines—In the above experiments, the Bₘₐₓ of HEK293 cells transiently transfected with the i3-1 mutant was lower than that in cells transfected
with WT BLT1 (Fig. 6). To confirm that the defects in Gi signaling in the i3-1 mutant were not due to decreased expression of the mutant receptor, we established monoclonal cell lines of WT BLT1 (lines 10 and 13) and the i3-1 mutant (lines 30 and 46) and determined $B_{\text{max}}$ values and signaling (Fig. 7). As shown in Fig. 7, A and B, the LTB$_4$ binding assay revealed that $B_{\text{max}}$ values for lines 10, 13, 30, and 46 of 8.1, 4.9, 7.6, and 14.4 pmol/mg protein, respectively. The $K_d$ values were 1.9, 1.5, 7.4, and 8.3 nM, respectively. Dose-response curves of the i3-1 mutants (lines 30 and 46) were shifted to the right by 1 order of magnitude compared with WT BLT1 (lines 10 and 13; Fig. 7C). Although the i3-1 mutant 46 was expressed higher than the WT BLT1 (lines 10 and 13), the IC$_{50}$ values of G$_i$-dependent adenylyl cyclase inhibition in the i3-1 mutant were higher than that in WT BLT1 (lines 10 and 13). However, a G$_{16}$-dependent IP accumulation was similar among cells expressing either WT or mutant receptors (Fig. 7D). These results further confirm the impaired Gi activation and normal G$_{16}$ activation.

**DISCUSSION**

Using mutagenesis studies, we show that three intracellular loops of BLT1 function in G-protein activation (Figs. 2B and 3B). Among these regions, we identified the i3-1 region as a selective activation site for Gi protein. The i3-1 mutant showed impaired Gi activation but normal G$_{16}$ activation (Figs. 4 and 7). The i3-1 region is located at the amino terminus of the third intracellular (i3) loop of BLT1. Previous reports suggest that the i3 region of several GPCRs functions in G-protein activation (27–30). In contrast, our findings show that the i3-1 (possibly i3-0) region of BLT1 is specifically required for Gi activation.

We used different cell lines for the different assays in the course of screening mutant receptors. We used HEK293 cells for GTP$_\gamma$S binding assay, because HEK293 cells
transiently expressing WT BLT1 showed higher LTB₄-induced GTP₇S binding than COS-7 cells. We used COS-7 cells for the IP accumulation assay, since Gaudreau et al. (20) had reported that COS-7 cells transfected with BLT1 and Go₁₆ showed LTB₄-induced IP accumulation. CHO stable cells are commonly used for GPCR research, because they are effective in examining intracellular signaling and cellular events, such as calcium mobilization, cAMP assay, and chemotaxis. Using these cells, we confirmed that the i3-1 mutant BLT1 exhibited impaired Go activation but intact G₁₆ activation.

LTB₄-dependent Go₂ activation as assessed by GTP₇S binding assay was impaired in the i3-1 mutant in HEK293 and CHO cells (Figs. 4A and 5, C and D). LTB₄-dependent inhibition of adenylyl cyclase and chemotaxis was also impaired in the i3-1 mutant in CHO stable cells (Fig. 5, E and F). However, LTB₄-dependent IP accumulation in COS-7 (Fig. 4B), HEK293 (data not shown), and CHO stable cells (Fig. 7G) expressing either the WT or i3-1 mutant was similar. These results show that impaired Go activation by the i3-1 mutant is not cell type-dependent.

We also isolated several monoclonal CHO cells expressing the WT or the i3-1 mutant BLT1 and performed similar experiments. Dose-response curves of LTB₄-induced adenylyl cyclase inhibition were shifted to the right in both high and low expresser of the i3-1 mutant compared with WT BLT1 (Fig. 7C). However, IP accumulations remained intact in the clones (Fig. 7D). We conclude that impaired Go activation by the i3-1 mutant is not dependent on the expression level of the receptor.

The binding studies showed that WT BLT1 exhibited high affinity for LTB₄ (Figs. 6 and 7, A and B). The i3-1 mutants and PTX-pretreated WT BLT1 showed only a low affinity binding state. In agreement with this data, Igarashi et al. reported that specific LTB₄ binding was increased by reconstitution with Ga proteins (Ga₁₆ (31)). Among them, reconstitution with Go₂ increased LTB₄ binding most efficiently. Banères et al. (32) showed that in the presence of Ga₂ proteins, BLT1 showed a high affinity binding state, using recombinant proteins produced in E. coli. In addition, Masuda et al. (33) reported that G₁-coupled BLT1 exhibited high affinity LTB₄ binding, whereas G₁₆-coupled BLT1 exhibited low affinity binding in a reconstitution system on baculovirus. These results and ours strongly suggest that Go coupling enables BLT1 to maintain a high affinity state, whereas BLT1 coupled to G₁₆ mediates a signal but exhibits only a low affinity state.

Pharmacological studies suggest the presence of high and low affinity binding sites for LTB₄ in spleen and granulocytes (34–36). Characterization of this low affinity binding site cannot be explained by the presence of BLT2, which constitutes a low affinity LTB₄ receptor (8–11), since human granulocytes do not express BLT2 (data not shown). Therefore, BLT1 might be responsible for low affinity binding of LTB₄ in neutrophils. The physiological relevance of the presence of low and high affinity binding states in BLT1 remains to be determined.
Banères and Parello (32) clearly showed that BLT1 was in a low affinity binding state in a reconstituted system completely devoid of any G-protein, whereas the addition of G-protein (Gαi2βγ2) binding induced a high affinity binding state. They also showed that homodimerized BLT1 associates with G-protein and forms BLT1-G-protein pentamers by using chemical cross-linking. Judging from the profiles in Western blotting analysis (Fig. 5B), it seems that the i3-1 mutant might be able to form a dimer, although more detailed studies will be required to confirm this (work in progress).

To gain insight into the molecular mechanism underlying how the i3-1 region of BLT1 recognizes G protein, we constructed a BLT1 three-dimensional model based on the structure of bovine rhodopsin (40) (Fig. 8) (19, 22). This model suggests that the i3-1 region might localize at the cytoplasmic end of helix V, not in the i3 loop of conventional seven-transmembrane models based on bacteriorhodopsin (Fig. 1A). The crystal structure of bovine rhodopsin shows that the cytoplasmic ends of helix V and VI are prominently extended below the plasma membrane in two different crystal forms, and these extended regions are highly flexible according to crystallographic temperature factors at more than 100 Å² (22, 37). In rhodopsin, it has also been suggested that this region of helix V and the membrane-proximal region of helix VI function in activation of transducin via a carboxyl-terminal region of Gαs (27, 38, 39).

We propose here that the BLT1 i3-1 region interacting with G protein may consist of the end of helix V in the model. Notably, alanine substitution in the i3-0 region, which contains three amino acid residues upstream of the i3-1 region, resulted in bluntness, alanine substitution in the i3-0 region, which contains three bulky hydrophobic (Tyr) residues.

In addition, the i3-0 region also contains two polar (Ser) and charged (Asp and Arg) residues with an amphiphilic (-helix destabilizing Gly residue interacting specifically with spherical and charged residues suitable for the proposed coupling to G-protein (41). In addition, the i3-0 region also contains two polar (Ser) and bulky hydrophobic (Tyr) residues.

The homology modeling of GPCRs based on bovine rhodopsin has been accepted for predicting and gaining insights into molecular basis of GPCR structure activity, although current homology modeling of GPCRs is not final or best structural solution. Combination of deduced model and functional experimental analysis have elucidated the molecular bases of GPCR structure activity, such as ligand binding and function of helix VIII (18, 19, 43). The i3 regions of BLT1 and bovine rhodopsin can be aligned automatically using the T-Coffee program (44).

This context, the three-dimensional model of the i3 region of BLT1 has confidence to some extent, although more detailed experiments are required to prove the above hypothesis in the future.

In summary, our work analyzes specific interactions between GPCRs and G-proteins using BLT1 as a model. These data suggest that BLT1 couples to both G16 proteins using overlapping intracellular region but that the i3-1 region functions only in G1 coupling, not in G16 coupling. The i3-1 mutant prefers G16 to G1 and maintains a low affinity LT4 binding state due to deficiencies in G1 coupling. This mutant receptor would be useful in determining specific signal transduction pathways mediated by G16 proteins via BLT1 and the biological significance of low and high affinity LT4 binding to BLT1. We propose that the helical structure of GPCRs acts as a switch for activation of G proteins. Examination of the roles of the cytoplasmic end of helix V of GPCRs other than BLT1 in determining specificity for coupling to G-proteins should be undertaken.

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