Recent studies have revealed that G-protein-coupled receptors contain a putative cytoplasmic helical domain, helix 8. Leukotriene B\(_4\) (LTB\(_4\)) receptor 1 derivatives with truncated or mutated helix 8 showed much higher LTB\(_4\) binding than wild-type (WT) receptors. Similar to the WT receptor, LTB\(_4\) promoted guanosine 5'\,-(3\,\text{thio})\,triphosphate (GTP\(^\gamma\)S) binding in these mutants. Unlike the WT receptor, however, the addition of GTP\(^\gamma\)S did not inhibit LTB\(_4\) binding to the mutant receptors. Scatchard analyses revealed that mutants maintained high affinity for LTB\(_4\) even in the presence of excess GTP\(^\gamma\)S. Consistently, mutant receptors showed a more prolonged Ca\(^{2+}\) mobilization and cellular metabolic activation than the WT receptor. From mutational studies and three-dimensional modeling based on the structure of bovine rhodopsin, we conclude that the helix 8 of LTB\(_4\) receptor 1 plays an important role in the conformational change of the receptor to the low affinity state after G-protein activation, possibly by sensing the status of coupling Go subunits as GTP-bound.

The activation of different classes of plasma membrane receptors regulates various biological functions in cells. The majority of these receptors belong to a superfamily of G-protein-coupled receptors (GPCRs), whose genes occupy more than 1% of the mammalian genes (1, 2). In general, ligand binding to GPCR results in a conformational change of the receptor that stimulates the activation of heterotrimeric G-proteins. All GPCRs are believed to share a common three-dimensional structure consisting of seven α-helical transmembrane (TM) domains connected by three intracellular (i\(_1\), i\(_2\), and i\(_3\)) and extracellular (e\(_\text{i}\), e\(_\text{a}\), and e\(_\text{j}\)) loops with intracellular C-terminal and extracellular N-terminal tails. The extracellular and transmembrane regions of the receptor are involved in ligand binding, whereas the intracellular surface is important for the activation of heterotrimeric G-proteins (3).

Recent studies reveal that the cytoplasmic tails of GPCRs have important roles, such as receptor trafficking, desensitization, dimerization, and intracellular signaling (4, 5). In the case of GABA\(_B\) (γ-aminobutyric acid type B) receptors, only heterodimers consisting of GABA\(_B\)_1 and GABA\(_B\)_2 subunits are functional (6–8). Surface expression of the assembled complexes is regulated through a dimerization-dependent trafficking checkpoint (9, 10). Studies have also shown that segments of the proximal C-terminal tail after the seventh transmembrane domain (TM7) in GPCRs are crucial for receptor transport to the cell surface. Naturally occurring truncations before TM7 of rhodopsin and vasopressin (V2) receptor result in endoplasmic reticulum-arsenated receptor transport, leading to the inherited diseases retinitis pigmentosa and nephrogenic diabetes insipidus, respectively (11). Truncations within the proximal C terminus of V2 and luteinizing hormone/choriogonadotropin receptors also impair their transport, whereas deletions of more distal portions have no effect (12, 13). Also, mutation of a Leu-Leu sequence in the proximal C terminus of V2 receptor impairs the transport to the cell surface (14). The C terminus of GPCR is also important in receptor desensitization. For example, the C termini of β\(_2\)-adrenergic receptor (15) and α\(_\text{MT}\)-adrenergic receptor (16) are phosphorylated by GPCR kinases, triggering desensitization of receptor-mediated responses. In the same manner, platelet-activating factor receptor (PAFR) internalizes after binding of PAF (17). Ligand-induced PAFR internalization depends on the association of C terminus of PAFR with arrestins (18).

Little structural information is available for GPCR and G-protein interactions. The only crystal structure that is known at the atomic level is that of rhodopsin, which was determined for the inactive form bound to the reverse agonist, 11-cis-retinal (19, 20). Analyses of the crystal structure of rhodopsin confirmed the presence of seven TM helices and revealed the existence of an eighth cytoplasmic helix (helix 8) that projects at a right angle from the C terminus of TM7. This short amphiphilic helix is anchored by palmitoyl groups to the cytoplasmic surface of the cell membrane. Marin et al. (21) show that mutations of three amino acids in N terminus of the helix 8 led to a dramatic decrease in the ability of rhodopsin to activate G\(_i\) (21), and a hypothetical model predicted that helix 8 interacted with the N-terminal helix of Go subunit (22).

Leukotriene B\(_4\) (LTB\(_4\)) (23), a metabolite of arachidonic acid, is a potent lipid mediator (24, 25). LTB\(_4\) mainly activates leuko-
hBLT1, a high affinity human LTB4 receptor, is mostly distinct ligand binding profiles and tissue distribution (28, 29). According to manufacturer’s instructions, cells were transfected with LipofectAMINE PLUS reagent (Invitrogen, Osaka, Japan). [3H]LTB4 (6016.2 GBq/mmol) and [35S]GTPγS release the ligand and signal shutdown. The helix 8 might work as a conformational changes of the receptor. The helix 8 of BLT1 is responsible for critical conformation (26). These actions of LTB4 are mediated by specific cell surface receptors, LTB4 receptors (BLTs) (27). Two GPCRs for LTB4 have been cloned in our laboratory and shown to have the epitope tag added at the N terminus. Sites of truncation are marked by bars. B, Edmundson helical wheel projection of the putative helix 8 region. The hydrophobic and hydrophilic amino acids tend to cluster on opposite faces of the wheel. C, C-terminal sequences of hBLT1 and the mutants. WT-hBLT1 encodes 352 amino acids. hBLT1dc323, -312, -305, -297, and -290 are truncated mutants of hBLT1. hBLT1 deletion is deleted with 8 amino acids (298–305). We substituted di-leucine at Leu-304 and Leu-305 for di-alanine in hBLT1AAA and Val-301 for alanine in hBLT1V301A.

kocytes and plays important roles in host defense and inflammation (26). These actions of LTB4 are mediated by specific cell surface receptors, LTB4 receptors (BLTs) (27). Two GPCRs for LTB4 have been cloned in our laboratory and shown to have distinct ligand binding profiles and tissue distribution (28, 29).

hBLT1, a high affinity human LTB4 receptor, is mostly expressed in leukocytes, with a little expression in thymus and spleen (29). hBLT1 is a small GPCR with 352 amino acids and a relatively long C-terminal tail of 64 amino acids (30). The proximal C terminus of hBLT1 contains a putative amphiphilic short helix, and this region is highly conserved among human, guinea pig (31), mouse, and rat BLT1 (32). Here we report that the putative helix 8 of BLT1 is responsible for critical conformational changes of the receptor. The helix 8 might work as a scaffold for G-proteins, changing it to the low affinity state to release the ligand and signal shutdown.

**EXPERIMENTAL PROCEDURES**

**Materials**—LTBs was a generous gift from Ono Pharmaceutical Co. (Osaka, Japan). [3H]LTB4 (6016.2 GBq/mmol) and [35S]GTPγS (46.2 TBq/mmol) were purchased from PerkinElmer Life Sciences.

**Mutagenesis of Human BLT1**—Hemagglutinin (HA)-tagged hBLT1 at N terminus was generated by PCR using a human BLT1 expression vector (pLTBR) (29) as a template. The oligonucleotide primers were 5’-ggagatccctagttcagttcgttttaacttg-3’ and 5’-ggatccctagttcagttcgttttaacttg-3’. The overlapping PCR method was used to generate the hBLT1 deletions hBLT1AAA and hBLT1V301A. The sequence of each construct was confirmed by DNA sequencing (model 3100; Applied Biosystems).

**Cell Culture, Transfection, and Flow Cytometry**—Human Embryonic Kidney (HEK) 293 and Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (Sigma-Aldrich), respectively, containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. These cells were transfected with LipofectAMINE PLUS reagent (Invitrogen) according to manufacturer’s protocol. After 48 h, the cells were fixed with PBS(−) containing 1% (v/v) paraformaldehyde for 5 min and blocked with PBS(−) containing 2% goat serum (Invitrogen) (PBS/GS) for 5 min. Then the cells were incubated with 2 μg/ml anti-HA antibody (3F10, Roche Diagnostics) or 2 μg/ml control rat IgG (Santa Cruz Biotechnology) in PBS/GS for 30 min, followed by staining with 10 μg/ml Alexa-Fluor-488-conjugated anti-rat IgG (Molecular Probes) for 30 min at room temperature. The cells were washed twice with PBS/GS and analyzed with a flow cytometer, EPICS XL (Coulter Electronics Ltd.). An EPICS ALTRA (Coulter Electronics Ltd.) was used for cell sorting.

**Ligand Binding Assay (Intact Cells)**—Ligand binding assays were performed using HEK293 or CHO cells plated in 24-well plates. 48 h after transfection, cells were washed with Heps-Tyrode’s BSA buffer (HTBSA, 25 mM Heps-NaOH, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 12 mM NaHCO3, 5.6 mM D-glucose, 0.37 mM Na2PO4, 0.49 mM MgCl2, and 0.1% (v/v) fatty acid-free BSA (Fraction V, Sigma-Aldrich)) and then incubated with 0.5 nM [3H]LTB4/HTBSA at room temperature for 1 h. After washing 3 times with HTBSA, cells were lysed with 1% Triton X-100, and the radioactivities were counted. For determination of nonspecific binding, at least a 1000 times higher concentration of unlabeled LTB4 was added to the binding mixture.

**Membrane Preparation and Western Blotting**—Cells were harvested, homogenized in ice-cold homogenize buffer (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 10 mM MgCl2, 2 mM EDTA, and protease inhibitor mixture (Sigma-Aldrich)) and centrifuged at 300,000 g for 1 h at 4 °C, and the resulting pellets were resuspended in the homogenize buffer. The protein concentration in each sample was measured by the Bradford method (protein assay kit, Bio-Rad). In some experiments, asparagine-linked (N-linked) carbohydrate moieties were removed with endoglycosidase F essentially according to the manufacturer’s instructions (Roche Diagnostics). Protein samples were separated in 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% skim milk in TBST (0.1% Tween 20 in Tris-buffered saline, pH 7.6), the blots were probed with 0.1 μg/ml anti-HA antibody (3F10, Roche Diagnostics) for 1 h. The membrane was then washed with TBST and incubated with 0.4 μg/ml horseradish peroxide-conjugated anti-rat IgG (Santa Cruz Biotechnology) for 1 h. The proteins were visualized using an ECL chemiluminescence detection system (Amersham Bioscience).
nonspecific binding (open squares) are presented (means ± S.D., n = 4). A and C are typical of at least three independent experiments.

Cell Enzyme-linked Immunosorbent Assay—Three × 10⁵ HEK293 cells well were transfected using LipofectAMINE PLUS reagent in 6-well plates. 24 h after transfection, the cells were replated into 96-well plates (2 × 10⁵ cells/well). After 48 h, cells were washed with HTBSA and then incubated with LTB₄ or PAF in HTBSA for 10 min, and the optical densities at 405 nm were measured with a microplate reader (Bio-Rad).

GTP-γ-S Binding Assay—The experiments were done as reported by Fukushima et al. (33) with slight modifications. The membrane proteins (16 µg) were incubated in 100 µl of GTP-γ-S binding buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 0.1% BSA, and 5 µM GDP containing 0.5 nM [³⁵S]GTP-γ-S) and various concentrations of LTB₄ for 30 min at 30 ºC. For determination of the nonspecific binding, unlabelled GTP-γ-S was added to the binding mixture to a final concentration of 20 µM. The bound [³⁵S]GTP-γ-S was separated from free [³⁵S]GTP-γ-S by rapid filtration through GF/C filters (Packard) and washed with 2 ml of ice-cold TMN buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 25 mM MgCl₂). The radioactivities of the filters were determined using a Top Count scintillation counter (Packard Instrument Co.).

Inhibition of LTB₄ Binding by GTP-γ-S—The membrane protein (12 µg) was incubated in 100 µl of LTB₄ binding buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 100 mM NaCl, and 0.05% BSA) containing 0.2 nM [³H]LTB₄, with various concentrations of GTP-γ-S. For determination of the nonspecific binding, unlabelled LTB₄ was added to the binding mixture to a final concentration of 1 µM. The mixtures were incubated for 1 h at room temperature followed by rapid filtration through GF/C filters and washing with 2 ml of LTB₄ binding buffer. The radioactivities were measured as described above.

Ca²⁺ mobilization assay—Nine × 10⁵ CHO cells were cultured on glass bottom dishes for 16 h and loaded with 3 µM Fura2/AM (Dojin, Kumamoto, Japan) in HTBSA buffer containing 0.01% (w/v) cresyl violet EL (Sigma) at 37 ºC for 1 h and further incubated at room temperature for 1 h. The cells were stimulated with ligands at room temperature. Fluorometric cell images were recorded with an ICCD camera/image analysis system (ARGUS-50, Hamamatsu Photonics) and the ratio (340 and 380 nm) was determined.

Cytosensor Microphysiometer Studies—Five × 10⁵ CHO cells were seeded into 12-mm Transwell inserts (3-µm pore size) and cultured for 16 h. Capsules were placed in sensor chambers and maintained by a flow (100 µl/min) of modified RPMI1640 (Molecular Devices). The flow was stopped for 40 s at the end of each 2-min pump cycle. The changes in the extracellular acidification rate were measured with the cytosensor microphysiometer (Molecular Devices).

hBLT1 Structure Modeling Based on Rhodopsin Structure—hBLT1 atomic model was constructed based on that of bovine rhodopsin with homology modeling as a rhodopsin sub-family member of GPCR (19). The amino acid sequences of these seven transmembrane proteins were aligned after the transmembrane sequence alignment in the GPCR data base (34). The O software (35) was used to replace amino acid residues according to the sequence alignment from bovine rhodopsin to hBLT1 on the atomic model of the bovine rhodopsin (PDB code 1F88) (19). Finally the structural idealization with molecular dynamics using the CNS software (36) was applied to the hBLT1 model. hBLT1 model constructed was checked by the Procheck software (37).

RESULTS

Expression and [³H]LTB₄ Binding Activity of WT-hBLT1 and the Mutants—To analyze the function of the hBLT1 C terminus we constructed serially deleted mutants of hBLT1 as shown in Fig. 1A. Edmundson wheel analysis predicts that the proximal C terminus of BLT1 forms an amphiphilic helix from Val-298 to Gly-307 (Fig. 1B). We constructed a hBLT1deletion that lacks 8 amino acids between Val-298 and Leu-305, hBLT1V30IA in which Leu-304–Leu-305 were substituted for Ala-Ala, and hBLT1V301A in which Val-301 was substituted for Ala (Fig. 1C). The functional properties of these mutants were tested by [³H]LTB₄ binding assay using intact cells after transient transfection with expression plasmids for the various mutant receptors (Fig. 2). We found that hBLT1de297 and hBLT1de290 showed much higher [³H]LTB₄ binding than WT-hBLT1 when transfected into HEK293 cells (Fig. 2A). Similar results were observed in CHO cells (Fig. 2B). hBLT1deletion and hBLT1V301A also showed much higher [³H]LTB₄ binding.

Calcium Mobilization Assay—Three × 10⁵ CHO cells were cultured on glass bottom dishes for 16 h and loaded with 3 µM Fura2/AM (Dojin, Kumamoto, Japan) in HTBSA buffer containing 0.01% (w/v) cresyl violet EL (Sigma) at 37 ºC for 1 h and further incubated at room temperature for 1 h. The cells were stimulated with ligands at room temperature. Fluorometric cell images were recorded with an ICCD camera/image analysis system (ARGUS-50, Hamamatsu Photonics) and the ratio (340 and 380 nm) was determined.
than WT-hBLT1, and hBLT1V301A showed relatively higher [3H]LTB4 binding than WT-hBLT1 (Fig. 2C). Expression levels of these receptors on the cell surface were analyzed by staining with anti-HA antibody specific to the N-terminal HA tag and Alexa-Fluor-488-conjugated secondary antibody followed by flow cytometry. Fig. 3A shows the results of flow cytometric experiments using HEK293 cells transfected with WT- and mutated hBLT1. Expression levels of the hBLT1 mutants were comparable with those of WT-hBLT1 judging from percentages of positively stained cells and the means of fluorescence intensity. The other mutants also showed similar expression (data not shown). Membrane fractions of HEK293 cells transfected with WT-hBLT1 and hBLT1dc290 were analyzed by Western blotting. WT-hBLT1 and hBLT1dc290 appear as diffuse and multiple bands (30–100 kDa) which are commonly observed for most GPCRs (Fig. 3B). After treatment with N-glycosidase F, these receptors migrated with the predicted molecular masses of 36 kDa. These results suggest that hBLT1 is glycosylated at one or both of two putative N-glycosylation sites (Asn-2 and Asn-164) presumably (Fig. 1A) and that expression levels of hBLT1dc290 were almost comparable with those of WT-BLT1. Surface expressions of WT-hBLT1 and hBLT1dc290 were also visualized by immunofluorescent staining with an anti-HA antibody, indicating that both WT and mutant receptors were expressed properly on the cell surface (Fig. 3C). We concluded that surface expression of WT-hBLT1 and the mutants was comparable.

We next determined whether the hBLT1 mutant internalizes more robustly than WT-hBLT1 after LTB4 stimulation. Greater internalization by mutant receptors could explain why the mutants exhibited higher [3H]LTB4 binding, i.e. more of the ligand becomes trapped inside the cells. To assess the receptor internalization cell surface receptors were quantified by cell-enzyme-linked immunosorbent assay before and after agonist stimulation. As previously described, PAFR rapidly internalized after exposure to 1 nM PAF, but not following exposure to LTB4 (Fig. 4A) (17). As shown in Fig. 4B, WT-hBLT1 and hBLT1dc290 did not internalize after 1 h exposure to LTB4 at concentrations as high as 100 nM. In similar experiments performed using HEK293 cells transiently transfected with these receptors, WT-hBLT1 and hBLT1dc290 did not internalize as well (Fig. 4C). Pretreatment with concanavalin A or 0.45 M sucrose, both of which are known to inhibit receptor internalization (38), did not alter the results of [3H]LTB4 binding assay using HEK cells expressing WT-hBLT1 and the mutants (data not shown). Thus, internalization could not explain the enhanced LTB4 binding to the mutants.

Roles of the hBLT1 C Terminus on G-protein Activation as Assessed by [35S]GTPγS Binding—Because G-proteins were reported to directly affect the structure and the ligand affinity
HEK293 cells were transiently transfected with WT-hBLT1, hBLT1dc290, and control cells were incubated with 0–100 nM LTB4, and analyzed as described in A. WT-hBLT1 (open circles), hBLT1dc290 (open triangles), and mock (open squares) are presented. Results are the means ± S.D., n = 4. C, HEK293 cells were transfected with WT-hBLT1, hBLT1dc290, and an empty vector. After 72 h, cells were washed by HTBSA, incubated with 0–100 nM LTB4, and analyzed as described in A. WT-hBLT1 (open circles), hBLT1dc290 (open triangles), and mock (open squares) are presented. Results are the means ± S.D., n = 4. All data are typical of at least three independent experiments.

Effects of GTPγS on [3H]LTB4 binding—We examined the effects of GTPγS, a hydrolysis-resistant GTP analogue, on [3H]LTB4 binding to membrane preparations from HEK293 cells transiently transfected with WT-hBLT1 or the mutants (Fig. 6). GTPγS caused a dose-dependent decrease in [3H]LTB4 binding to WT-hBLT1. In contrast, [3H]LTB4 binding to the hBLT1 mutants was inhibited only weakly by GTPγS as high as 1 mM. These results indicate that the hBLT1 mutants can exchange GDP for GTP with G-protein, but the mutants do not change the affinity for [3H]LTB4 even after G-protein activation.

Scatchard Analyses of WT-hBLT1 and hBLT1dc290 in the Presence or Absence of GTPγS—We performed Scatchard analyses using membrane preparations from HEK293 cells transiently transfected with WT-hBLT1 or hBLT1dc290 (Fig. 7). Ten-μg membrane protein aliquots were incubated with various concentrations of [3H]LTB4 in the presence or absence of 100 μM GTPγS. The addition of GTPγS apparently decreased [3H]LTB4 binding to WT-hBLT1 (Fig. 7, A and B). The Kd values of WT-hBLT1 for [3H]LTB4 changed from 0.84 ± 0.09 to 4.46 ± 0.66 nM by the addition of 100 μM GTPγS, whereas the Bmax value was not affected (0.64 ± 0.05 to 0.62 ± 0.03 pmol/mg of protein in the absence and presence of 100 μM GTPγS, respectively) (Fig. 7C). These results indicate that WT-hBLT1 changed from the high affinity state to low affinity in the presence of GTPγS. On the other hand, the addition of GTPγS did not decrease [3H]LTB4 binding to hBLT1dc290 compared with WT-hBLT1 (Fig. 7, D and E). The Kd values of hBLT1dc290 were 1.64 ± 0.12 and 2.84 ± 0.77 nM in the absence and presence of 100 μM GTPγS, and the Bmax values were 1.58 ± 0.11 pmol/mg of protein and 1.72 ± 0.31 pmol/mg of protein, respectively (Fig. 7F). These results indicate that hBLT1dc290 maintains high affinity for LTB4 even at high concentrations of GTPγS, whereas WT-hBLT1 clearly changed to low affinity state.

Longlasting Signal of the hBLT1 Mutants—To examine LTB4-induced intracellular signaling, we established CHO cells stably expressing WT-hBLT1, hBLT1dc290, or hBLT1LLAA. After 72 h the cells were harvested, and the membrane fractions were recovered. The membrane protein (12 μg) was incubated in LTB4 binding buffer with 0.2 nM [3H]LTB4, and 0–1000 μM GTPγS for 1 h at room temperature. The bound radioactivities to the membrane preparations were measured. hBLT1 (open circles), hBLT1dc290 (open triangles), hBLT1deletion (filled circles), and hBLT1LLAA (filled triangles) are presented. Results are typical of two independent experiments.
To avoid the problems of clonal variations, drug-resistant cells were sorted as polyclonal populations by fluorescence-activated cell sorter, and the sorted cells were analyzed by flow cytometry. As shown in Fig. 8A, WT-hBLT1, hBLT1dc290, and hBLT1LLAA were expressed at similar levels. We measured LTB4-induced intracellular calcium mobilization in these CHO cells (Fig. 8B). In CHO-WT-hBLT1 cells, LTB4 induced a rapid increase of intracellular calcium concentration that immediately decreased. Although LTB4 induced a rapid increase of intracellular calcium concentration similar to CHO-WT-hBLT1, the calcium concentration decreased very slowly in CHO-hBLT1dc290 and CHO-hBLT1LLAA cells. Intracellular calcium concentration remained at high levels even 4 min after washout of LTB4. CHO-Mock cells did not respond to LTB4, whereas CHO-Mock, WT-hBLT1, hBLT1dc290, and hBLT1LLAA cells responded similarly to 10 nM ATP (data not shown).

We next performed cytosensor microphysiometer studies to measure the cellular metabolic activity by monitoring the accumulation of hydrogen ions, since activated cellular metabolism by ligand stimulation acidifies extracellular fluid. As shown in Fig. 8C, CHO-WT-hBLT1 cells acidified the medium only transiently after exposure to 100 nM LTB4. By contrast, CHO-hBLT1dc290 and CHO-hBLT1LLAA cells acidified the medium for prolonged periods of time after exposure to LTB4. All of the transfected CHO cells responded only transiently after exposure to ATP. These results indicate that the hBLT1 mutants cannot properly shut down the switch-on signal after LTB4 depletion.

hBLT1 Structure Model Based on Rhodopsin Structure—To elucidate the molecular mechanism of the hBLT1 function, we constructed a molecular model of hBLT1 based on that for bovine rhodopsin as a reference. Rhodopsin was chosen since it is the only GPCR for which the structure is known at the atomic level (19). Because hBLT1 belongs to the same sub-subfamily (class 1a; GPCRDB: www.gpcr.org/7tm) as rhodopsin, the sequence alignment readily allowed us to construct a preliminary model. We refined this model to account for the specific properties of hBLT1 using a software for protein model building and a molecular dynamics software.

Our hBLT1 model predicts the presence of a cytoplasmic helical segment (helix 8) extending from TM7 similar to the one observed in rhodopsin. Fig. 9 shows a close-up of the residues in the vicinity of helix 8. The hBLT1 model suggests that a pair of aromatic residues (Tyr-285 and Phe-300), which are positioned similarly to a conserved Tyr-306 and Phe-313 pair in rhodopsin, may stabilize the inactive form of the receptor by holding TM7 and helix 8 in almost a right angle to each other. Hydrophobic amino acids (Val-301, Leu-304, and Leu-305) of amphiphilic short helix 8, which were mutated in this study, may anchor the helix to the plasma membrane. A phosphorylation site (Thr-308) is located just after the amphiphilic helix 8, and
Fig. 8. Agonist-induced calcium mobilization and acidification of WT-hBLT1 and the mutants. A, CHO cells were transfected with empty vector, HA-WT-hBLT1, HA-hBLT1dc290, or HA-hBLT1LLAA. The transfected cells were sorted by cell sorter and analyzed by flow cytometry with anti-HA antibody and Alexa-Fluor-488-conjugated second antibody. Means of fluorescence intensity is 0.58 in Mock, 82.0 in...
Role of Helix 8 in the Activated BLT1

Fig. 9. hBLT1 structure model building on bovine rhodopsin in ribbon. The hBLT1 atomic model was constructed based on that of bovine rhodopsin (PDB code 1F88) with homology modeling as a rhodopsin sub-family member of GPCR; the vicinity is presented in the color green; helix 8 is in *violet*, transmembrane helix 7 is in *pink*, and helix 1 is in *blue*. Helix 8 with side chains in a stick model was shown. In helix 8, the hydrophobic side chains of Val-301, Leu-304, and Leu-305 are colored in *violet* and extrude to the plasma membrane, and a pair of aromatic residues, Tyr-285 and Phe-300, with a transparent Corey-Pauling-Koltun surface may restrain helices 7 and 8 as a clasp like Tyr-306 and Phe-315 in bovine rhodopsin structure.

the phosphorylation there can be predicted to weaken the interaction between helix 8 and the plasma membrane. Phosphorylation at that site may also play a role the inactivation of receptor signaling by recruiting (40). Helix 8 of hBLT1 may function as a scaffolding domain for G-proteins and may regulate receptor conformation. Our studies of mutant receptors suggest that helix 8 is more important for maintaining and recovering the inactive conformation.

**DISCUSSION**

Although GPCRs respond to diverse agonists, the structural and functional features of receptors are remarkably conserved (41, 42). As was first elucidated for rhodopsin and β2-adrenergic receptor, GPCRs contain seven membrane-spanning domains, an extracellular N terminus, and an intracellular C terminus. In response to stimulatory ligands, GPCRs undergo a conformational change, promote the exchange of GDP for GTP on the G-protein α-subunit, and initiate the dissociation of the G-protein α- and βγ-subunits. Subsequently, the activated Go- and Gβγ-subunits positively and/or negatively regulate the activity of effector enzymes and ion channels (43, 44).

After G-protein activation, GPCRs are inactivated by several mechanisms. The best-characterized mechanism of inactivation involves the phosphorylation of intracellular Ser/Thr residues of GPCRs by GPCR kinase, protein kinase C, and cAMP-dependent protein kinase, which leads to β-arrestin-dependent internalization. On the other hand, in a ligand binding assay using membrane preparations GPCRs switch to a low affinity state after the addition of GTPγS. However, no information concerning the molecular mechanism of this conformational change is currently available. Using various mutants of hBLT1, we found that the putative helix 8 plays an important role in the conformational change of the receptor to the low affinity state after G-protein activation.

BLT1 is the high affinity receptor for LTB4 and plays an important role in host defense and inflammation (27). Studies using mice with a disrupted BLT1 gene confirm that BLT1 functions in acute inflammation and immediate hypersensitivity as well as in leukocyte activations (45, 46). The BLT1-signaling pathway involves activation of phosphoinositide-specific phospholipase Cβ via Bordetella pertussis toxin (PTX)-sensitive (G<sub>i</sub>) and PTX-resistant (G<sub>G</sub>, G<sub>q</sub>) G-proteins (29, 47).

Desensitization of LT<sub>B</sub> receptor after LT<sub>B</sub> stimulation was reported mainly in leukocytes. Pretreatment with protein kinase C activators inhibited LT<sub>B</sub> binding to human neutrophils (48) and the coupling of G-proteins to LT<sub>B</sub> receptor in porcine neutrophils (49). In vivo desensitization has also been demonstrated in rabbit neutrophils after LT<sub>B</sub> exposure (50). Recently, Gaudreau et al. (40) report that Thr-308 of hBLT1 is a major target residue involved in GPCR kinase 6-mediated desensitization.

Rhodopsin is the best-studied GPCR and the only one whose three-dimensional structure is known at the atomic level (19). One of the novel features that emerged from the rhodopsin crystal structure was the helical conformation (helix 8) of the proximal cytoplasmic region, contiguous to the cytoplasmic membrane. Because BLT1 belongs to the rhodopsin subfamily of GPCRs, we hypothesized that the proximal amphiphilic region of hBLT1 also forms a helix 8. An Edmundson helical wheel projection for this region is shown in Fig. 1B. A molecular model for hBLT1 based on the three-dimensional structure of rhodopsin readily accommodated a cytoplasmic helix 8 (Fig. 9). The crystal structure of rhodopsin revealed that amino acid side chains of helix 8 interact with those of helix 1 and helix 7 (20) and the C terminus of the α- and γ-subunits of G<sub>i</sub> (44, 51). The distances between helix 8 and other parts of rhodopsin significantly change before and after activation (52, 53).

To study the functional role of hBLT1 C terminus, we constructed various mutants of hBLT1 (Fig. 1) and found that hBLT1dc307 and hBLT1dc290 showed much higher [3H]LTB<sub>4</sub> binding than WT-hBLT1 and hBLT1dc305 when transiently transfected to HEK293 cells (Fig. 2A) and CHO cells (Fig. 2B). We hypothesized that a segment of amino acid residues between 297 and 305 might be important for ligand binding. To test this idea we constructed hBLT1deletions hBLT1LLAA and hBLT1V301A (Fig. 1C), based on the Edmundson wheel analysis (Fig. 1B) and performed [3H]LTB<sub>4</sub> binding assay using cells in which they were expressed (Fig. 2C). Surprisingly, hBLT1deletion and hBLT1LLAA showed increased [3H]LTB<sub>4</sub> bindings over hBLT1dc297, and hBLT1V301A showed a relatively higher [3H]LTB<sub>4</sub> binding than WT-hBLT1. Next, we analyzed the expression levels of the receptor protein by flow cytometry, Western blotting, and confocal laser microscopy (Fig. 3). All results indicate that WT-hBLT1 and the mutants are expressed on the cell surface at a comparable level. The enhanced [3H]LTB<sub>4</sub> binding to the mutant receptors could not be explained by the enhanced internalization of the receptors, since WT-hBLT1 and the mutant did not internalize at all in our system (Fig. 4).

WT-hBLT1, 49.6% in hBLT1dc290, and 89.6% in hBLT1LLAA. B, CHO cells stably expressing WT-hBLT1, hBLT1dc290, or hBLT1LLAA were loaded with Fura2/AM and stimulated with 100 nM LTB<sub>4</sub> for 30 s and then washed-out. The image at 150 s is before stimulation, the image at 180 s is just stimulated, and the 400 s image is 4 min after stimulation. The ratio of fluorescence intensities was recorded with an Argus image analyzing system as described under “Experimental Procedures.” C, CHO cells stably expressing WT-hBLT1, hBLT1dc290, or hBLT1LLAA were stimulated with 10 nM ATP or 100 nM LTB<sub>4</sub> for 1 min and then washed-out. Extracellular acidification rates were measured using a four-channel Cytosensor Microphysiometer. Data are typical of at least three independent experiments.
GPCRs alter their conformation from the high affinity state to low affinity after exchange of GDP for GTP on the coupled G-proteins. To quantify the ability of the receptors to activate G-protein, we examined LTB₄-stimulated [³⁵S]GTP₅S binding to the G-protein using the membrane fractions of the cells transfected with the receptors. WT-hBLT1 and mutant receptors exhibited a similar dose-dependent increase in [³⁵S]GTP₅S binding (Fig. 5), showing that mutant receptors induced exchange of GDP for GTP on G-proteins as well as WT-hBLT1. Next, we examined the effects of GTP₅S on [³⁵H]LTB₄ binding to WT and mutant receptors (Fig. 6). The addition of GTP₅S decreased [³⁵H]LTB₄ binding to WT-hBLT1 in a dose-dependent manner. In contrast, [³⁵H]LTB₄ binding to the mutant receptors (hBLT1dc290, dc297, deletion, and LLAA) was inhibited only weakly by GTP₅S as high as 1 μM. Furthermore, we performed Scatchard analyses using WT-hBLT1 or hBLT1dc290. Although hBLT1dc290 changed to low affinity on the presence of GTP₅S (Fig. 7C), hBLT1dc290 exhibited a minimal change by GTP₅S addition (Fig. 7F). These results suggest that hBLT1dc290 is resistant to the conformational change of the receptor to the low affinity state after G-protein activation. By binding assay using intact cells (Fig. 2A), hBLT1dc290 exhibited a 20-fold increase in LTB₄ binding over WT-hBLT1. However, the difference was only severalfold using the membrane fractions (Fig. 7). Although the clear explanation of the difference between intact cells and membranes is unclear, the following possibilities are present. 1) Cytoplasmatic GTP content is inhibitory for the binding of LTB₄ to the WT-hBLT1 but not to the mutants, as suggested by Fig. 6; 2) intracellular signaling activates various kinases (GPK kinase, protein kinase C, etc.) and inactivates the receptor by phosphorylating the C terminus.

To examine LTB₄-induced intracellular signaling we established CHO cells stably expressing WT-hBLT1 or the mutants at a similar level by cell sorting (Fig. 8A). In CHO-WT-hBLT1 cells, LTB₄ induced a rapid and transient increase of intracellular calcium concentration. CHO-hBLT1dc290 and LLAA exhibited sustained calcium increase comparing to the transient increase in CHO-WT-hBLT1 (Fig. 8B). Similarly, although CHO-WT-hBLT1 only transiently induced extracellular acidification, CHO-hBLT1dc290 and LLAA showed prolonged acidification after exposure to LTB₄. The reported interaction of Gₐ and helix 8 of rhodopsin and the three-dimensional modeling of hBLT1 (Fig. 9) suggested that helix 8 of hBLT1 transduces the signals from the GTP form of Go subunit to the receptor. Alternatively, the helix 8 of hBLT1 is necessary for the conformational change of the receptor to the low affinity state as an anchor to the plasma membrane.

When only the mutant receptors were introduced the constitutive activities were not observed in GTP₅S binding in Fig. 5 and in basal calcium concentrations in Fig. 8B. We performed several experiments on the constitutive activities of the mutants. When the mutants were expressed together with Go₁₂ in HER293 cells, the constitutive activities were observed in GTP₅S binding (data not shown). This may be explained by the low quantity of the intrinsic G-proteins in the cells.

In conclusion, we propose that the helix 8 of hBLT1 plays an important role in the conformational change of the receptor after G-protein activation from the mutational studies and the predicted three-dimensional structure of hBLT1. Although we have to await the final conclusion until the three-dimensional structure of GPCR-G-protein complex is shown, the present study has shown that a helix 8 of hBLT1 is involved in a certain step in detecting the signals from the GTP form of Go subunit and subsequent conformational change of the receptor to release the ligand and shut down the intracellular signaling.

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