Critical Role for Polar Residues in Coupling Leukotriene B₄ Binding to Signal Transduction in BLT1*

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Leukotriene B₄ (LTB₄) mediates a variety of inflammatory diseases such as asthma, arthritis, atherosclerosis, and cancer through activation of the G-protein-coupled receptor, BLT1. Using in silico molecular dynamics simulations combined with site-directed mutagenesis we characterized the ligand binding site and activation mechanism for BLT1. Mutation of residues predicted as potential ligand contact points in transmembrane domains (TM) III (H94A and Y102A), V (E185A), and VI (N241A) resulted in reduced binding affinity. Analysis of arginines in extracellular loop 2 revealed that mutating arginine 156 but not arginine 171 or 178 to alanine resulted in complete loss of LTB₄ binding to BLT1. Structural models for the ligand-free and ligand-bound states of BLT1 revealed an activation core formed around Asp-64, displaying multiple dynamic interactions with Asn-36, Ser-100, and Asn-281 and a triad of serines, Ser-276, Ser-277, and Ser-278. Mutagenesis of many of these residues in BLT1 resulted in loss of signaling capacity while retaining normal LTB₄ binding function. Thus, polar residues within TM III, V, and VI and extracellular loop 2 are critical for ligand binding, whereas polar residues in TM II, III, and VII play a central role in transducing the ligand-induced conformational change to activation. The delineation of a validated binding site and activation mechanism should facilitate structure-based design of inhibitors targeting BLT1.

Seven transmembrane receptors widely known as G-protein-coupled receptors (GPCRs)§ (1, 2) mediate an array of physiological processes in response to such diverse agonists as peptides, amino acid derivatives, and lipids. Despite the great diversity in their ligands, the conserved motifs found across this superfamily and the limited interacting partners such as G-proteins (3) and β-arrestins (4) at the cytoplasmic interface point toward a common activation mechanism for GPCRs. GPCRs constitute the single largest group of molecules for drug targets due to their critical importance in mediating biological responses as well as their easy accessibility on the cell surface. However, very little structural information is available for GPCRs due to difficulties in purifying and obtaining crystal structures for this class of receptors.

The availability of the rhodopsin crystal structure (5) combined with the approach of computational modeling and validation by site-directed mutagenesis has led to delineation of ligand-receptor interactions in a few GPCRs (6–9). Some elements of the activation mechanism have been identified for individual GPCRs (1, 6, 8, 10–12). Several studies employing site-directed mutagenesis have helped uncover critical interactions between residues in transmembrane domains of the GPCRs (reviewed in Ref. 1). The approach of computational modeling with validation by site-directed mutagenesis has led to significant increases in the understanding of the processes involved in GPCR activation (6–9).

Leukotriene B₄ (LTB₄) is a potent leukocyte chemoattractant and mediates its biological effects through two distinct GPCRs, the high affinity receptor BLT1 and the low affinity receptor BLT2 (13, 14). Several recent studies suggested a direct and critical role for BLT1 in diverse inflammatory diseases such as arthritis (15, 16), atherosclerosis (17, 18), and asthma (19). Recently, the high affinity LTB₄ receptor, BLT1, was expressed in Escherichia coli and shown to form a functional pentameric complex with heterotrimeric G-proteins (20). Computational modeling has been used to investigate the potential role of the eighth helix in signaling of BLT1 (21, 22). In addition, a recent study reported an LTB₄ binding site in BLT1 deduced from computational models (23). However, the exact nature of the LTB₄ binding site and the potential changes in receptor conformation following LTB₄ binding remain unknown.

In this study, computational modeling together with site-directed mutagenesis led to precise mapping and validation of the LTB₄ binding site in BLT1. Mutation of each of the residues predicted to be in the putative binding site resulted in reduced binding affinity. Furthermore, analysis of dynamic structures of the ligand-free and ligand-bound BLT1 allowed prediction of critical movements of transmembrane helices and essential
interhelical interactions stabilizing both the active and inactive states of the receptor. This analysis uncovered an activation core centered around Asp-64 (D2.50; according to the Balles-teros-Weinstein numbering system) (11) and is composed of polar amino acids Asn-36 (N1.50), Ser-100 (S3.35), Ser-277 (S7.45), Ser-278 (S7.46), Ser-279 (S7.47), and Asn-281 (N7.49) and led to the formulation of an activation mechanism. The deduced mechanism of BLT1 activation was consistent with the experimental observations made with several mutants of activation core residues.

**EXPERIMENTAL PROCEDURES**

**Homology Modeling and Identification of LTB₄ Binding Site in BLT1**—The multiple alignment of the human LTB₄ receptors, BLT1 and BLT2, with bovine rhodopsin was generated using ClustalW (24). The alignment was in agreement with the known literature on helix stabilization motifs, disulfide linkage conservation, and core-forming residue conservation in GPCRs (25) and was used to generate homology models based on the crystal structure of bovine rhodopsin (5) (PDB id: 1F88) as a template in Modeler (26). The homology model of BLT1 was energy-minimized (hydrogen atom addition was carried out by using xleap and minimized using 5,000 steps of steepest descent followed by 20,000 steps of conjugate gradient using AMBER8 (27) sander with ff99) and then used for docking with LTB₄. A structure for LTB₄ was generated in SYBYL7.0 (Tripos Inc., St. Louis, MO), and charges and potentials were assigned to the receptor (Kollman All) and ligand (gasteiger). A consensus docking approach was used wherein multiple docking algorithms were employed to improve the scoring process. LTB₄ was docked into BLT1 using DOCK (28), SURFLEX (29), and AUTODOCK (30). The consensus structure for ligand-docked BLT1 was refined using restricted molecular dynamics using the SYBYL7.0 dynamics module, employing the AMBER 7 FF99 for 500 ps with a 1-fs time step. The “aggregates” option was employed to keep frozen (fixed) all the parts of the receptor except the ligand, and the ligand binding zone was defined as having a radius of 8 Å with the ligand as center. The resulting structure was then energy-minimized (5,000 steps of steepest descent followed by 20,000 steps of conjugate gradient), and molecular dynamics simulations in vacuo were carried out (100,000, 25 ps followed by 300,000 for 125 ps with positional restraints of 1000 kcal (mol Å⁻¹) using AMBER 8 (27) (sander ff99) on the entire receptor, excluding the above defined binding pocket residues and the ligand, which were left unrestrained to further optimize contacts and relax the pocket. The binding mode was visualized using Insight II (Accelrys Inc.) and SYBYL7.0. The figures were generated with Insight II, Mole-script (31), and Raster3D (31).

**Molecular Dynamics Simulations**—Molecular dynamics simulations of the ligand-bound and ligand-free forms of the receptor were carried out in a lipid bilayer solvated system consisting of lipid, water, and ions using previously reported protocols (32). Specifically, xleap was used to generate the solvated lipid bilayer system, comprising the homology model of BLT1, 375 pre-equilibrated dodecyl maltoside lipid molecules, 10,111 TIP3P solvent molecules, and Cl⁻ ions added for neutrality. Our standard equilibrium and production-run protocols were used (32). Molecular dynamics simulations were performed with the AMBER-99 force field using AMBER 8.0, and the following: Message Passing Interface Sander module in the isothermal isobaric ensemble (p = 1 atmosphere) and anisotropic pressure scaling (ntp = 2); periodic boundary conditions with PME; and 1.5-fs time step. Hydrogen atoms were frozen using SHAKE. The ligand was parameterized with an antechamber program (33) using the general AMBER force field and HF/6–31G*-derived RESP atomic charges from GAMESS. The production runs were unrestrained and were carried out for 5 ns using 32 Opteron processors. The same protocol was followed for BLT1 with and without the bound natural agonist, LTB₄.

Molecular dynamics trajectories were analyzed via energy versus time and root mean square deviation (of the backbone atoms) versus time for the post equilibration production phase. The analysis established the energetic and structural stability of the system for both ligand-free and ligand-bound BLT1 simulations.

**Site-directed Mutagenesis**—The construction of cDNA of hemagglutinin epitope-tagged BLT1 was described previously (34). A red fluorescent protein (RFP)-monomer was tagged at the C terminus of BLT1 by in-frame cloning of the entire coding region of the receptor without a stop codon ahead of the RFP-coding region in pDsRed-monomer N1 vector (BD Biosciences 632465). Site-specific mutants of BLT1-RFP-monomer were generated using the PCR-based sense/antisense primer method (35), and all mutants were confirmed by DNA sequencing.

**Expression of Receptors and Generation of Clonal Stable Cell Lines**—Functional properties of the native and mutant receptors were examined in 300.19 cells. 300.19 is a murine pre-B cell line with no detectable endogenous expression of any LTB₄ responsive receptors and was previously used to study the signal transduction pathways activated by leukocyte adhesion molecules (36) and chemokine receptors (37). 300.19 cells were maintained in RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 55 µM 2-mercaptoethanol and transfected by electroporation. The stable single cell-derived cell lines with BLT1 or its mutants were selected by FACS (Moflo) and maintained in the presence of 1 mg/ml G418. The cell surface expression was determined by incubating parental cells or cells expressing different receptor variants with 12CA5 antibody followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG and analyzed using FACSCalibur (BD Biosciences).

**Ligand Binding**—Whole cell competition ligand binding (34) or saturation binding (13) assays were performed with 300.19 cells expressing BLT1 or its mutants. For competition binding assay, the cells (0.5 × 10⁶ per assay) were incubated with 2.5 nM [³H]LTB₄ (0.25 nM for BLT1) (163 Ci/mmol, PerkinElmer Life Sciences) along with increasing concentrations of cold ligand (Cayman Chemicals, Detroit, MI) in binding buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM NaCl, 0.05% bovine serum albumin (fatty acid-free Fraction V, Sigma A8806)). These mixtures were incubated on ice with gentle agitation for 2 h followed by rapid filtration through GF/C filters (Whatman 1822-025) using manifold-vacuum setup and washed with 3 ml of ice-cold binding buffer. The radioactivity of the filters was determined with a Beckman Coulter LS6500 multipurpose
scintillation counter. Nonspecific binding was determined by inclusion of 2 μM unlabeled LTB₄ to the cells suspended in 2.5 nM [³H]LTB₄. The competition curves were generated using non-linear regression fit on GraphPad Prism software, and the EC₅₀ were determined from these curves and expressed as nanomolar and binding sites/cell, respectively. Similar values were obtained from Scatchard analysis of the data.

Chemotaxis and Calcium Measurements—Migration of 300.19 cells was evaluated using 5-μm pore size Transwell filters (Corning Costar, Cambridge, MA). Cells (1 × 10⁷) were resuspended in 1 ml of chemotaxis buffer (RPMI 1640, 1% fetal bovine serum). The lower chamber was loaded with various concentrations of LTB₄ in a volume of 600 μl, and 100 μl (1 × 10⁶ cells) of cells from the above cell suspension was placed onto the upper chamber. After 3 h of incubation at 37 °C in 5% CO₂, the upper chamber was removed, and cells in the lower chamber were counted in a Bürker chamber. Calcium mobilization was monitored in INDO-I-loaded cells (300.19 cells expressing BLT1-WT or its mutants) stimulated with various concentrations of LTB₄ as previously described (34).

Receptor Phosphorylation—300.19 cells (5 million cells per sample) expressing BLT1-RFP or its mutants were serum-starved for 2 h in 5 ml of phosphate-free Dulbecco’s modified Eagle’s medium with 20 mM HEPES, pH 7.0. Cells were washed with the same buffer and labeled with [³²P]orthophosphate (150 μCi per sample, 8500–9120 Ci/mmol, PerkinElmer Life Sciences) in the above buffer in a total volume of 1.5 ml for 1.5 h, and cells were stimulated for 5 min at 37 °C with LTB₄ at concentrations indicated. Cells were washed twice with ice-cold 1× phosphate-buffered saline (without Ca²⁺ and Mg²⁺) containing 0.1% bovine serum albumin. The harvested cells were lysed with 1 ml of radiolmmune precipitation assay buffer (50 mM Tris-Cl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM NaF, 1 mM sodium pyrophosphate, and protease mixture inhibitor tablet from Roche Applied Science). Hemagglutinin-tagged receptors were immunoprecipitated with high affinity anti-hemagglutinin antibody (clone 3F10 from Roche Applied Science). Immunoprecipitates were resolved by 10% SDS-PAGE, and one site binding (hyperbola) non-linear regression curve fitting (GraphPad Prism 4.0). Kₐ and Bₘₐₓ were determined from these curves and expressed as nanomolar and binding sites/cell, respectively. Similar values were obtained from Scatchard analysis of the data.

FIGURE 1. Leukotriene B₄ binding site in human BLT1. A, schematic representation of human BLT1: The boundaries of each transmembrane, extracellular, and intracellular domains are derived from the molecular dynamics modeled structure of BLT1. The amino acid residues involved in the ligand binding and activation process are shaded green and red, respectively. Mutated residues in ECL2 showing no effect on the ligand binding or signaling are shaded in pink. B, LTB₄ docked structural model for BLT1 refined by restricted molecular dynamics (“Experimental Procedures”): The seven transmembrane helices are colored: TM1 (blue), TM2 (green), TM3 (yellow), TM4 (brown), TM5 (gray), TM6 (pink), TM7 (orange), and helix VIII (red). LTB₄ is rendered as space-filled with carbon atoms shaded dark gray, hydrogen atoms in white, and oxygen atoms in red. C, the binding groove with LTB₄ bound. Surface rendering of a cross-section of BLT1 displaying the binding site with LTB₄ docked (ligand represented as thick sticks, carbon atoms are green, oxygen atoms are red, and hydrogen atoms are white).
dried gels were exposed to phosphorimaging screens and analyzed on a Typhoon 9400 variable-mode gel imager (Amersham Biosciences). Integrated counts were evaluated using ImageQuaNT software (Amersham Biosciences).

BLT1 was subjected to minimization and in vacuo molecular dynamics allowing both the receptor binding pocket and the ligand to relax and attain stable conformations of lowest energies. A snake diagram depicting the primary sequence of

Real-time Fluorescence Microscopy—RBL-2H3 cells were co-transfected with β-arrestin-GFP and either with wild-type or mutant receptor tagged with RFP (monomer), and images were captured as described previously (18, 34).

RESULTS

Identification of LTB4 Binding Site in BLT1—Bovine rhodopsin had been the only available crystal structure (5) for a GPCR and hence heavily relied upon for homology modeling-based approaches for structural studies of GPCRs. The rhodopsin ligand (11-cis-retinal) is covalently linked to the receptor, whereas LTB4 is a mostly hydrophobic lipid with three nodes of polarity. The polar head group is a carboxylate, which can form hydrogen bonding or strong ionic interactions, and the hydroxyl groups at the 5th and 12th carbon positions are also capable of forming hydrogen bonding interactions.

To obtain a starting structure for BLT1, a sequence alignment of human BLT1 with bovine rhodopsin (1F88) was generated. This alignment was then manually modified to precisely match the known motifs in the transmembrane (TM) regions for rhodopsin with those of BLT1. A homology-based three-dimensional structural model for human BLT1 that included a conserved disulfide linkage between Cys-90 and Cys-168 (25, 38) was generated and energy minimized. A “consensus docking protocol” that used three independent docking algorithms, AUTODOCK (30), SURFLEX (29), and DOCK (28), was employed to obtain a ligand-docked structure for BLT1. The top three ranked results from each of the docking runs were analyzed, and the highest common factor or the most common binding mode was selected as a starting binding mode for LTB4 in BLT1. This structure of LTB4 docked into}

FIGURE 2. Characterization of LTB4 binding site mutants in BLT1. A, BLT1 ligand binding pocket. Computational model of the BLT1 binding pocket with LTB4 bound, obtained by a consensus docking protocol ("Experimental Procedures"). The predicted hydrogen bonding and electrostatic interactions are shown for Glu-185, Tyr-102, Asn-241, Arg-156, Thr-157, His-94, and LTB4, which stabilize the bound ligand in the pocket. Transmembrane helices III, IV, V, and VI and ECL2 are shown with TMV rendered transparent. Hydrogen atoms are not displayed for clarity. B, stable expression of BLT1 and its mutants in 300.19 cells. Indirect immunofluorescence staining of 300.19 cells (gray) and 300.19 stably expressing BLT1 and the indicated mutants with 12CA5 mAb and fluorescein isothiocyanate-conjugated goat anti mouse IgG. C and D, saturation ligand binding. Representative saturation binding curve and Scatchard plot of [3H]LTB4 binding to the 300.19 cells expressing BLT1 and Y102A. Data shown are representative of at least two independent experiments with triplicate measurements in each experiment. E, competition ligand binding. Representative curves from one of three independent experiments of [3H]LTB4 competition binding to the 300.19 cells expressing BLT1 or the indicated mutants. E, chemotaxis. Ligand-dependent chemotaxis of BLT1 or its mutants was measured by Transwell filters as described under “Experimental Procedures.” Data are the mean ± S.D. of cells counted from two individual wells (four fields were counted per well) that migrated to the lower chamber for each concentration from a representative experiment of at least three repetitions. The color scheme in panels B, E, and F is as follows: black, BLT1; orange, Y102A/N241A; red, Y102A; blue, N241A; purple, E185A; and cyan, H94A.
BLT1 is shown in Fig. 1A. The residues identified as ligand contact points are marked in green. The structure of LTB₄ bound to BLT1 is shown in Fig. 1B. The ligand binding site of BLT1 is composed of ECL2 and TMs III, V, and VI, and the hydrophobic ligand penetrates deep into the transmembrane domains. The three nodes of polar regions in the ligand are countered by corresponding polar residues in the receptor. A clear ligand binding pocket is visible in the surface rendering of the LTB₄ binding site of BLT1 (Fig. 1C).

**LTB₄ Interaction with Residues in TM Domains**—The hydrophobic nature of the ligand suggested a deep seated pocket within BLT1. Analysis of the ligand-bound structure identified several potential contact points in the TMs: His-94 and Tyr-102 (TM III), Glu-185 (TM V), and Asn-241 (TM VI), whereas the polar head group was oriented toward ECL2 (Fig. 2A). Both Glu-185 (TM V) and Tyr-102 (TM III) stabilize LTB₄ binding by direct hydrogen bonding with the C5/H₁₁₀₃₂ hydroxyl group as well as with each other, whereas Asn-241 (TM VI) hydrogen bonds to the hydroxyl group on C₁₂/H₁₁₀₃₂ of LTB₄ at the base of the binding pocket. His-94 likely interacts with the C⁵-hydroxyl of LTB₄ via hydrogen bonding.

To validate these interactions, receptor variants were generated by mutagenesis. Amino acid residues His-94, Tyr-102,
Asn-241, and Glu-185 were mutated to alanine in a combination of single and double mutants. All these receptor variants were stably expressed to similar levels as single cell derived lines on the surface of 300.19 cells (Fig. 2B). All the mutants displayed reduced levels of binding as determined by both competition and saturation binding assays (Fig. 2, C–E, and Table 1). The four single mutants H94A, Y102A, N241A, and E185A displayed reduced binding affinity of 4- to 9-fold in competition binding and 6- to 15-fold in saturation binding assays (Table 1). Surface expression as measured by flow cytometry or as $B_{\text{max}}$ in saturation binding assays also showed that all mutants are expressed at comparable or higher levels relative to BLT1 (Table 1). The Y102A/N241A double mutant showed a synergistic reduction in binding affinity in $[^{3}H]$LTB$_{4}$ binding assays (Fig. 2E and Table 1). LTB$_{4}$ activates a variety of cellular responses, including chemotaxis (14), calcium mobilization, and ligand-induced phosphorylation and desensitization (39). Consistent with the reduction in binding, all the mutants showed a shift in dose response of chemotaxis (Fig. 2F) while retaining the bell-shaped nature of the response. The Y102A/N241A double mutant required 100 times more LTB$_{4}$ for reaching the maximum response. Analysis of ligand-induced calcium mobilization also showed a clear shift in dose response with each of these mutants (Fig. 3). The Y102A, H94A, and N241A mutants and the Y102A/N241A double mutants reached similar levels of maximum activity but at much higher concentrations than the wild-type receptor (Fig. 3). A summary of the functional properties of all the mutants is shown in (Table 1).

A Critical Role for ECL2 in LTB$_{4}$ Binding—The ligand-docked structural model showed Arg-156 and Thr-157 being located on ECL2, which caps the binding site on top of the TM helix bundle. A charge interaction between Arg-156 and the carboxylate group of LTB$_{4}$ was predicted to stabilize LTB$_{4}$ in the binding pocket. The model also predicted Thr-157 as being within hydrogen bonding distance of the carboxylate head group of LTB$_{4}$. The other possible charge interactions on ECL2 were with Arg-171 and Arg-178. To delineate the relative contribution of these residues to ligand binding, the R156A/T157A, R171A/R178A, R156A, and T157A mutants were stably expressed to similar levels in 300.19 cells (Table 1). The R156A/T157A and the R156A mutants failed to show any $[^{3}H]$LTB$_{4}$ binding, whereas the T157A and R171A/R178A mutants displayed similar ligand binding affinity to that of BLT1 (Table 1). Consistent with the complete lack of LTB$_{4}$ binding the R156A/T157A and the R156A mutants did not show a detectable chemotactic response (Fig. 4A). In contrast, both the T157A and R171A/R178A mutants showed a similar bell-shaped chemotaxis response as BLT1 (Fig. 4A). Likewise, ligand-induced calcium mobilization by all these mutants followed a similar profile with R156A/T157A and the R156A mutants showing a minimal response and T157A and R171A/
R178A showing a similar response to that of BLT1. Thus, Arg-156 is a critical residue for LTB₄ binding and, as a consequence, for signaling in BLT1.

**Ligand-induced Phosphorylation of BLT1 Mutants**—Concomitant with activation, ligand binding induces a dose-dependent phosphorylation of the GPCRs by GPCR kinases (40) leading to desensitization of signaling. Previous studies have shown that BLT1 displays a basal level of phosphorylation that is enhanced by treatment with LTB₄ (34). Fig. 5 shows that BLT1 undergoes rapid LTB₄ dose-dependent phosphorylation, and all the binding site mutants displayed ligand-induced phosphorylation but required higher LTB₄ concentrations to be fully phosphorylated. The RT mutant while displaying basal phosphorylation did not show any further increase in receptor phosphorylation at any concentration of LTB₄.

**Inactive and Active States of BLT1**—An experimentally validated ligand binding mode in BLT1 described above formed the basis for examining the ligand-induced conformational changes in BLT1. Lipid bilayer molecular dynamics simulations were run for the ligand-free and ligand-docked homology modeled structures of BLT1 to generate optimized inactive and active state models. A comparison of the models of the ligand-free and ligand-bound states of BLT1 by superposition revealed movement of transmembrane helices (Fig. 6, A and B) as well as conformational changes at the amino acid level (see below). TMs I, II, and IV showed minimal movement, TMs III and V showed moderate movement, and TMs VI and VII showed the largest transmembrane helical movement (Fig. 6, A and B). The large conformational changes observed in TMs VI and VII of BLT1 are consistent with similar changes associated with the activation of rhodopsin (41–43).

**Identification of an Activation Core in BLT1**—Hydrogen bonding, Van der Waals, and electrostatic interactions were analyzed for both the inactive and active states of BLT1. A cluster of polar residues comprising Asn-36, Asp-64, Ser-100, Asn-281, Ser-277, Ser-278, and Ser-279 was found to differentially stabilize the inactive and active states of BLT1 and form the activation core (Fig. 7, A and B). In both the inactive (Fig. 7A) and active states (Fig. 7B) Asp-64 was the hub of the interactions. In the inactive state, Asp-64 was hydrogen-bound with Ser-100 (d = 2.6 Å) and Ser-278 (d = 2.7 Å). The Ser-100-Asp-64 and Ser-278-Asp-64 interactions were conserved in both the inactive and active states of BLT1 (Fig. 7, A and B). During activation, Asn-281 and Asn-36 moved upward by 3.2 Å and 7 Å (Fig. 7B), respectively, to hydrogen-bond with Asp-64. These interactions are completely absent in the inactive state (Fig. 7A).

To determine the validity of this activation mechanism each of these polar residues were mutated to alanine, and the mutant receptors were stably expressed to similar levels in clonal lines of 300.19 cells (Fig. 7C and Table 2). All of these mutants displayed ligand binding affinity similar to the wild-type BLT1 both in competition and saturation binding assays (Table 2) with the exception of the S277A/S278A/S279A mutant that bound LTB₄ with reduced affinity (Table 2). Saturation binding analysis also showed that each of the mutants is expressed at similar or higher levels relative to BLT1 (Table 2).

Analysis of the activation core mutants for chemotaxis, calcium mobilization, ligand-induced phosphorylation, β-arrestin association, and internalization revealed severe reduction in their activity relative to BLT1. First, all mutants displayed
reduced chemotaxis with N281A showing a complete loss of response (Fig. 7D). D64A and S277A/S278A/ S279A showed a shift as well as a reduction in response. Despite the significant reduction in magnitude, all mutants showed a bell-shaped chemotaxis curve indicating normal gradient sensing by these mutants.

Dose-dependent calcium mobilization also showed a severe reduction in response for many of these mutants (Fig. 8, A–D). Although the maximum response reached for the D64A mutant was only ~25% that of the BLT1 response, other mutants reached responses of 60–100% to that of native BLT1. However, much greater inhibition was seen at the EC90 of the BLT1 response, where for most mutants the response ranged from 0 to 10%. In particular, the D64A and N281A mutants showed very little response at 1.0 and 10 nM LTB4 (Fig. 8, B and C, and Table 2). Thus, despite similar binding affinity for LTB4, these BLT1 mutants fail to convert ligand binding into an effective cytoplasmic signal. Simultaneous with receptor activation, ligand binding affects dose-dependent phosphorylation of GPCRs by GPCR kinase (40) leading to desensitization as well as association of the phosphorylated receptors with β-arrestin.

BLT1 and all the activation core mutants showed a basal level of receptor phosphorylation as well as a comparable increase in phorbol 12-myristate 13-acetate-induced phosphorylation (44) (Fig. 8, E and F). Although BLT1 showed a robust increase in ligand-induced phosphorylation, most activation core mutants displayed relatively weak ligand-induced phosphorylation (Fig. 8, E and F). Consistent with the complete loss of chemotaxis response and severe reduction in calcium mobilization, the N281A mutant showed no ligand-induced phosphorylation but was a substrate for phorbol 12-myristate 13-acetate-induced phosphorylation. Translocation of cytoplasmic β-arrestin to the membrane is a ubiquitous phenomenon following

FIGURE 6. Molecular models of active and inactive states of BLT1. Superimposition of the predicted inactive (blue) and active (green) state structural models of BLT1 are shown. A, lateral view of the structure of BLT1 outlining major helical movements along the vertical (y) axis. B, top view showing rotation of helices around the y-axis vector and translation of helices in the x-z plane. Helix VIII and loops are not shown for clarity. LTB4 is rendered as space-filled with carbon atoms in cyan, hydrogen atoms in gray, and oxygen atoms in red.
Glycoprotein receptor activation (45). Fig. 8G shows the typical rapid β-arrestin translocation following LTB₄ treatment of cells expressing BLT1-RFP and β-arrestin-GFP. In this assay N281A failed to interact with arrestin, whereas the other mutants displayed relatively weak or delayed association of β-arrestin with RFP-receptors (Fig. 8G). Thus, despite similar binding affinity for LTB₄, these BLT1 mutants fail to convert ligand binding into an effective cytoplasmic signal.

**DISCUSSION**

Computational models identified the potential contact points for LTB₄ in BLT1 that were validated by mutational analysis. Molecular dynamics analysis of the ligand−free and ligand-bound structural models of BLT1 predicted essential residues involved in differentially stabilizing the inactive and active states of the receptor via critical polar interactions. Experimentally, these were proven to affect the signaling mechanism supporting a conceptual activation core in class A GPCRs. A high degree of conservation of the polar activation core residues across the class A GPCRs lends further support for the mechanism.

The use of multiple docking protocols and molecular dynamics of the binding pocket allowed the delineation of the LTB₄ binding site in BLT1. Experimental results with mutants support the accuracy of the predicted binding mode, validating this approach. The hydrophobic nature of LTB₄ dictates to a large extent its deep seated binding mode in the BLT1 pocket. The ligand is encompassed by transmembrane domains III, V, and VI, whereas ECL2 forms a lid over the pocket. Each of the three TMs contributes residues toward ligand binding (His-94 and Tyr-102 in TM III, Glu-185 in TM V, and Asn-241 in TM VI). The three nodes of polarity in LTB₄ are being countered by four polar residues in the receptor, and mutation of each had a measurable effect on binding affinity and signaling. The N241A, Y102A, E185A, and H94A mutants and the Y102A/N241A double mutant each attained the wild-type maximum levels of signaling in functional assays, including chemotaxis, calcium flux, and receptor phosphorylation, albeit at a much higher concentration of LTB₄ as compared with BLT1. This suggests that, although these residues located in TMs III, V, and VI contribute to the binding affinity by countering the hydroxyl groups at 5th and 12th positions on LTB₄, they are not absolutely critical for LTB₄ binding or ligand-induced conformational changes associated with receptor activation. In contrast, interaction of the carboxylate head group with ECL2 is absolutely critical for binding. Whereas both Arg-156 and Thr-157 were predicted to be involved in ligand binding, analysis of double and single mutants established the critical role of Arg-156 in LTB₄ binding. Mutation of arginine 156 to alanine resulted in minimal levels of signaling as expected from the complete loss of ligand binding. These findings suggest a more critical role for ECL2 residues in LTB₄ binding than that for the transmembrane domains. The negative charge of the carboxylic head group of LTB₄ is effectively countered by the positively charged arginine residue (Arg-156). The ECL2 may be involved in initial ligand entry as well as being critical for ligand stabilization via a hinged lid-like action closing and completing the binding pocket. An integral role for ECL2 in ligand binding has also been reported for other GPCRs (46, 47).

A recent study reported a molecular modeling-based binding pocket for LTB₄ in BLT1 (23). The study concludes with the identification of Arg-178 and Glu-185 as residues involved in ligand receptor interactions and Val-105 and Ile-108 as lining the binding pocket. In general, the overall binding modes described here and in Sabirsh et al. (23) are similar in that the orientation of the ligand along the receptor axis, with the hydrophobic tail seated deep in the core and the polar head group pointing toward the extracellular surface. Secondly, the Glu-185 was identified as a ligand binding residue by both models via interaction with the C5′′-hydroxyl group of LTB₄.

However, the binding mode proposed here differs from Sabirsh et al. with respect to identification of the residues involved in ligand stabilization, the magnitude of the effects of point mutations on ligand binding, and the experimental and computational strategies employed to arrive at these conclusions. The studies here identify and validate His-94, Tyr-102, Asn-241, and Arg-156 as residues involved in LTB₄ binding to BLT1, whereas none of these residues were considered by Sabirsh et al. (23). In contrast, they have indicated Arg-178 as a binding site residue based on the R178L mutation. The magnitude of the effect on ligand binding observed in Sabirsh et al. upon mutating Arg-178 to Leu could possibly be a result of the energetic penalty arising out of replacing a polar residue by a highly hydrophobic residue. In our study, mutating Arg-178 to alanine had no effect on ligand binding. In contrast, it is the Arg-156 on ECL2 that is most critical for LTB₄ binding as evidenced by complete loss of binding in the R156A mutant while being expressed normally on the cell surface. Alanine substitution was used as a mutagenesis strategy for all our analysis as this would minimize other indirect effects.

**TABLE 2**

| Mutant | Surface expressiona | EC₅₀ b | Kᵦ c | B_max c | Calcium released |
|--------|---------------------|--------|------|---------|-----------------|
|        | %                   |        |      |         |                 |
| BLT1   | 100                 | 3.4    | 1.2  | 17,518  | 100             |
| N281A  | 177                 | 3.6    | 1.4  | 52,877  | 14.8            |
| D64A   | 219                 | 8.2    | 2.9  | 36,998  | 0.3             |
| N36A   | 111                 | 2      | 1.2  | 48,201  | 103.6           |
| S277A/S278A/S279A | 126     | 32.8   | 7.1  | 29,754  | 23.58           |

a Relative surface expression of BLT1 (100%) and mutants (Fig. 7C).
b EC₅₀ measured from competition [³H]LTB₄ binding assays. Data are representative of at least three independent experiments with triplicate measurements in each.
c Kᵦ and B_max measured from saturation binding assays. Data are representative of at least two independent experiments with triplicate measurements in each.
d % of the maximum calcium response at 10 nM LTB₄ (Fig. 8D).

**LTB₄ Binding Site and Activation of BLT1**
LTB₄ Binding Site and Activation of BLT1

A

![Graph showing ratio over time for BLT1 with different LTBA concentrations](image)

B

![Graph showing ratio over time for D64A](image)

C

![Graph showing ratio over time for N281A](image)

D

![Graph showing % Maximum over LTBA concentrations for BLT1, N36A, S277-79A, D64A, and N281A](image)

E

![Images of Western blot for BLT1, N36A, D64A, and S277-79A](image)

F

![Graph showing phosphorylation fold over basal for BLT1, N36A, D64A, S277-79A, and N281A](image)

G

![Images of immunofluorescence for BLT1, N36A, D64A, S277-79A, and N281A over time](image)
TABLE 3
Conservation of polar residues in class A GPCRs

Included are the frequency of occurrence of the polar residues across 1636 class A GPCRs (www.GPCR.org) and the percent conservation at congruent positions to that of BLT1 (residue numbers shown in italics) and activation core (The BLT1 residue types are in bold).

| Residues     | Type and positiona |
|--------------|--------------------|
|             | N1.50 (N36) | D2.50 (D64) | S7.45 (S277) | S7.46 (S278) | S7.47 (S279) | N7.49 (N281) |
| Asn          | 1636       | 46         | 504         | 32          | 9           | 1027         |
| Ser          | 15         | 221        | 489         | 111         | 59          |
| Cys          | 0          | 8          | 146         | 441         | 6           |
| His          | 0          | 115        | 1           | 7           | 2           |
| Asp          | 1495       | 4          | 17          | 8           | 6           |
| Glu          | 44         | 11         | 13          | 28          | 6           |
| Gln          | 1          | 15         | 19          | 13          | 12          |
| Thr          | 3          | 209        | 113         | 43          | 67          |
| Tyr          | 3          | 10         | 14          | 33          | 30          |
| Arg          | 0          | 33         | 12          | 17          | 15          |
| Lys          | 1          | 22         | 26          | 17          | 16          |
| Polar residues | 1636      | 1608       | 1152        | 882         | 727         | 1453         |
| Total residues | 1636      | 1636       | 1636        | 1636        | 1636        |
| % Conservation | 100       | 98.3       | 70.4        | 53.9        | 44.4        | 88.8         |

a Positions are shown in parentheses.

The differences in the structural models may be accounted for by the contrasting computational strategies employed. Although Sabirsh et al. based their model of BLT1 on a theoretical active state model of rhodopsin; our models were based on the crystal structure of inactive state bovine rhodopsin (PDB id: 1F88). In our studies, LTB₄ was successfully docked into the inactive state of BLT1 in a minimum energy conformation following a consensus docking approach. It is important to note that docking LTB₄ into a lipid bilayer-simulated structure of BLT1 in itself was insufficient to accurately identify all the interactions. Further, the use of fully solvated lipid bilayer molecular dynamics simulations might better approximate the receptor microenvironment as compared with energy minimized homology models developed by Sabirsh et al. (23).

A few of the residues identified in this study as being critical to LTB₄ binding in BLT1 have been implicated in mediating ligand binding via congruent positions in other class A GPCRs. The Glu-185 residue in BLT1 finds a parallel in the Glu-182 residue of the histamine binding site of the histamine H₄ receptor (48). Similarly, an equivalent of the His-94 residue (TM III) has been shown to play a critical role in ligand recognition in the human A₃ adenosine receptor as His-95 (49). The formylmethionyleucylphenylalanine binding site in its receptor was dissected using a chimeric approach and revealed the essential role of extracellular loops in ligand binding (50). An essential role for Arg-156 (ECL2) in ligand binding in BLT1 was established in this study. Interestingly, arginines in ECL2 were shown to be important in ligand binding in several other chemoattractant receptors. In prostaglandin D₃ receptor (CRTH2) R178A mutation in ECL2 resulted in a 5-fold decrease in the binding affinity (51). In CCR5, Arg-168 in ECL2 is essential for binding MIP1α (52). Similarly, it has been shown in the C5a receptor that arginine 175 is an important counterion in binding C5a. In CXCR3, arginine 197 and arginine 212 in ECL2 are critical for ligand binding, whereas arginine 216 is critical for receptor activation but not for ligand binding or internalization (53). Given the chemical diversity of the GPCR ligands, these examples highlight the role of arginines in ECL2 in ligand binding in GPCRs.

The residues Tyr-102, Arg-156, Glu-185, and Asn-241 are all conserved in the low affinity LTB₄ receptor BLT2, but the His-94 is replaced by Tyr in BLT2. Despite the limited homology between BLT1 and BLT2 (~45%), conservation of most of the binding site residues described here suggests a common binding mode for LTB₄ in BLT1 and BLT2. The difference in the binding affinities between BLT1 and BLT2 for LTB₄ may thus be related to the replacement of His-94 with Tyr and other possible changes in helical orientation and/or interaction distances between the key binding residues in BLT2. Application of similar strategies described here should allow precise identification and rationalization of the LTB₄ binding site in BLT2. The elucidation of the LTB₄ binding site renders BLT1 amenable to a structure-based drug design.

The BLT1 structures with and without bound LTB₄ allowed delineation of the ligand-induced conformational changes leading to a preliminary activation mechanism. In the generalized toggle switch activation mechanism (54) of GPCRs, TM VI (55) and VII (42) are known to move outwards at the extracellular face of the receptor. Similar helical movements were...
observed for BLT1 activation, and the final alignments of TM VI and VII were consistent to both those in rhodopsin (12, 41, 55) and in the generalized toggle switch mechanism. Upward and downward movements of the helices resulted in an increased cytoplasmic penetration of intracellular loops 2 and 3 in the active state of the receptor. This, along with a more open arrangement of the TMs at the cytoplasmic face, is consistent with the need for increased surface area for interaction with signaling partners such as G-proteins and β-arrestin. The model for BLT1 is also consistent with respect to only minimal changes being seen for TMs I–IV (54).

A unique element of the current study is that molecular dynamic simulations were predictive and allowed rationalization of global changes in receptor conformation to altered interactions at the level of a few amino acid residues. These residues couple the ligand binding domain to signaling via appropriate conformational changes. The receptor variants generated by site-directed mutagenesis of the activation core residues allowed uncoupling of ligand binding function from signaling function. Both D64A and N281A mutants bind LTB4 with the same affinity as BLT1 yet activate minimal signaling functions. Thus, reorientation of helix VII bringing the Asn-281 in juxtaposition to Asp-64 appears a critical event for stabilizing the active state of BLT1. The interaction of Asp-64 and Asn-281 has been implicated as being critical in several other GPCRs (1, 6, 8). An interesting difference in the gonadotropin-releasing hormone (56) receptor is that Asp-64 and Asn-281 are replaced by Asn-87 and Asp-318. The residues when reversed at this position retained wild-type receptor functions, whereas single mutants were defective in ligand binding. In the case of the serine triad Ser-277–79 the interactions were more complex, because Trp-234 stabilizes the inactive state via hydrogen bonds with Ser-278 but switches to Ser-277 in the active state. In the serine triad mutant loss of stabilizing forces both in the active and inactive states of the receptor might have resulted in the observed phenotype of both reduced binding affinity as well as signaling capacity.

The general applicability of the proposed BLT1 mechanism to other class A GPCRs was examined in 1636 sequences from the GPCR data base (www.gPCR.org). The activation core residues at positions Asn-36, Asp-64, Ser-100, Ser-277, Ser-278, Ser-279, and Asn-281 occur at a frequency of Asn-36 (100%), Asp-64 (91.25%), Ser-100 (<1%), Ser-277 (13.5%), Ser-278 (29.9%), Ser-279 (6.8%), and Asn-281 (64%). At the "SSS" locus, depending on the receptor subclass there exist other common motifs for GPCRs such as HCC, NSC, and NSS and other such combinations of polar residues. Of the 1636 GPCRs analyzed nearly 100% have conserved polar residues at positions equivalent to Asn-36 and Asp-64, whereas other positions also showed high levels of polar residue conservation across class A GPCRs (Table 3). Thus, at congruent positions to those identified in BLT1, conservation of polarity rather than exact identity of the residues might be critical for GPCR function (57, 58). It is possible that each of these conserved positions in other GPCRs represent similar elements of the activation mechanism as seen for BLT1.

The polar residues comprising the ligand binding site and activation core are critical in coupling LTB4 binding to signal-

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