Molecular Dissection of the Interaction between hBLT2 and the G Protein Alpha Subunits

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Leukotriene B4 (LTB4) is a potent chemoattractant for leukocytes and considered to be an inflammatory mediator. Human BLT2 (hBLT2) is a low-affinity G-protein coupled receptor for LTB4 and mediates pertussis toxin-sensitive chemotactic cell movement. Here, we dissected the interaction between hBLT2 and G-protein alpha subunits using GST fusion proteins containing intracellular regions of hBLT2 and various Gα protein including Gαi1, Gαi2, Gαi3, Gαs1, Gαo1, and Gαz. Among the tested Gα subunits, Gαi3 showed the highest binding to the third intracellular loop region of hBLT2 with a dissociation constant (K_D) of 5.0×10^{-6} M. These results suggest that Gαi3 has the highest affinity to hBLT2, and the third intracellular loop region of hBLT2 is the major component for the interaction with Gαi3.

Key Words : Leukotriene B4, hBLT2, G-protein, Intracellular loop, GPCR-G protein interaction

Introduction

Leukotriene B4 (LTB_4), generated from arachidonic acid via the 5-lipoxygenase pathway, is a lipid inflammatory mediator of the recruitment and activation of leukocytes.[1] It exhibits strong chemotactic effects on neutrophils[2] as well as eosinophils.[3,4] Although LTB4-mediated leukocyte recruitment has a protective role against various pathogens,[5,6] it is also involved in multiple inflammatory pathologic conditions, including rheumatoid arthritis,[7] asthma,[8] and inflammatory bowel disease.[9] Leukotriene-deficient mice are deficient in their response to inflammatory stimuli.[10] Therefore, antagonists for LTB4 receptors are in development for therapeutic purposes.[11-13] LTB4 mediates its function through G-protein coupled receptors (GPCR). There are two human GPCRs for LTB4 (hBLT1 and hBLT2) which have strong sequence homology (45.2% amino acid identity) with each other and the corresponding mouse genes.[14,15]

Human BLT2 is a low-affinity LTB4 receptor, which has an approximately 100-fold lower affinity (K_D = 20 nM) than that of hBLT1 (K_D = 0.22 nM).[16] It possesses pharmacologically different properties than hBLT1 since a number of hBLT1 antagonists do not inhibit LTB4 binding to hBLT2.[17] Moreover, hBLT2 is expressed relatively ubiquitously in human tissues,[16] whereas hBLT1 is expressed predominantly in leukocytes,[18] suggesting that hBLT2 mediates cellular functions in tissues other than leukocytes. In addition, hBLT2 is implicated in the cell transformation or oncogenic pathway. The expression of hBLT2 is enhanced in transformed cells by oncogenic Ha-Ras,[19] and LY293111, an antagonist of hBLT2, inhibits the proliferation of human pancreatic cancer cells.[20] Hence, blockade of LTB4/hBLT2 and the downstream signaling pathway is assumed to be a potential target for the treatment of cancer as well as inflammatory disease.

The signaling pathway of hBLT2 was investigated by measuring the cellular levels of cAMP or Ca^{2+} in CHO cells expressing hBLT2.[16] The effect of LTB4 on the transformed CHO cells with hBLT2 exhibited increased intracellular calcium concentration as well as induced chemotactic responses, indicating that hBLT2 is coupled to both the Gi and Gq families of G-proteins. When the cells were pretreated with pertussis toxin (PT), the chemotactic response of the hBLT2-transformed cells by LTB4 was completely blocked, indicating that a member of the Gi family mediates the chemotactic responses. In contrast, the increase of intracellular calcium by LTB4 in hBLT2-transformed cells was only partially blocked by treatment with PT, suggesting that the calcium response was mediated both by PT-sensitive and PT-insensitive G proteins or hBLT2 couples to different types of G-proteins. In the case of hBLT1, its signaling pathway could be mediated by various types of G proteins. Activation of hBLT1 by LTB4 inhibits the formation of cAMP through PT-sensitive G-proteins,[21] and cotransfection of hBLT1 with Gα16 in Cos-7 cells results in an enhanced production of inositol phosphate in response to LTB4,[22] suggesting that the LTB4-dependent activation of phospholipase C involves G16 in addition to Gi. Although hBLT1 and hBLT2 have been coupled with more than two types of G proteins, as yet the specificity of hBLT1 and hBLT2 to various G proteins has not been completely analyzed. Also, the PT-sensitive Gi protein(s) responsible for hBLT2-mediated chemotaxis has yet to be uncovered.

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In this study, we prepared various Gα subunits and GST fusion proteins containing intracellular regions of hBLT2, and investigated the relative affinities of the GST fusion proteins to the Gα subunits of the Gi family (Gi1, Gi2, Gi3, Go1, or Gz) as well as Gs1 to identify the Gi protein(s) critical for hBLT2-mediated chemotactic mobility. We showed that the third intracellular loop region of hBLT2 is the critical region for the interaction with the Gαi3 protein by characterization of the interaction between and Gαi3 and GST fusion proteins.

Materials and Methods

Construction of expression vectors for GST fusion proteins and the Gα subunits. The oligonucleotides encoding the second intracellular region (iL2, amino acids 118-141) and the third intracellular region (iL3, amino acids 206-225) of hBLT2 were ligated at the C-terminus of GST in pET22b to produce pGST-iL2_{BLT2} and pGST-iL3_{BLT2}. Also, the C-terminus tail of hBLT2 (CT, amino acids 287-358) was amplified from pcDNA3-hBLT2 harboring the cDNA of hBLT2 (Gene bank accession number: AB029892), and ligated at the C-terminus of GST in pET22b to produce pGST-CT_{BLT2}. The DNA fragment encoding the open reading frame of the human Gαi3 was amplified by PCR from human fetal liver cDNA (Novagen, USA), and the clones Gαi1, Gαi2, Gαo1, Gαs1 and Gαz were obtained from the Human Gene Bank (Korea Research Institute of Bioscience and Biotechnology, Korea). The genes coding Gαi1, Gαi2, Gαi3, Gαo1 and Gαz were amplified with specific primers and cloned into a pET28a vector to produce Gα proteins contain a 6X-His tag at the N-terminus.

Preparation of GST fusion proteins and the Gα subunits labeled with a fluorescence probe. GST, GST-iL2_{BLT2}, GST-iL3_{BLT2}, and GST-CT_{BLT2} were expressed in *E. coli* BL21(DE3) cells. The expression of GST or GST-fusion proteins were induced by the addition of 1 mM of IPTG when the optical density of culture at 600 nm reached 0.7, and cells were harvested after 4 hr of growth at 37 °C. The harvested cells were disrupted by ultrasonication in 25 mL buffer A (50 mM sodium phosphate, 0.15 M NaCl, pH 7.4, and 1 mM PMSF and 1 mM EDTA) with 80 mg of Complete Mini Cocktail inhibitor (Roche Diagnostics, Germany). The lysate was centrifuged at 13,000 rpm and the supernatant was loaded onto a glutathione affinity resin column (Peptron Inc. Korea), which was pre-equilibrated with buffer A. After washing thoroughly, the GST fused proteins were eluted using 20 mM reduced glutathione in buffer A. The glutathione in the purified protein sample was removed by desalting column and it was stored at −80 °C for further analysis. The purified GST fusion proteins were dialyzed against buffer A and stored at −80 °C.

The N-terminus histidine tagged Gα (s1, i1, i2, i3, o1 and z) proteins which were expressed in *E. coli* Rosetta 2 (DE3). Purification of His-tagged Gα proteins was performed as described previously.[23] Briefly, expression of these proteins was induced by the addition of 1 mM of IPTG when the optical density of the culture at 600 nm reached 0.7, and cells were harvested after 16 hr of growth at 18 °C, except for Gαz, which was expressed at 24 hr at 12 °C. All the Gα proteins, except Gαz, were at least 30-40% in the soluble form. Less than 5% of the expressed Gαz protein was recovered as soluble protein. The cells were lysed by ultrasonication in 25 mL buffer C (50 mM Tris-HCl, 0.15 M NaCl, 20 mM β-mercaptoethanol and 1 mM PMSF, pH 7.4) and centrifuged at 13,000 rpm to remove the cell debris and insoluble proteins. The cleared lysates were loaded onto a Ni^{2+}-charged chelating column pre-equilibrated with buffer C. The His-tag labeled Gα proteins were eluted with 0.3 M imidazole in buffer A and labeled with a fluorescence probe according to the manufacturer's manual (Sigma) using flourescein isothiocyanate (FITC).

Measurement of the interaction between GST fusion proteins and the Gα subunits. The interaction of GST, GST-iL2, GST-iL3, GST-CT with FITC-Gα proteins were analyzed by measuring the amount of bound Gα protein on immobilized GST fusion proteins. GST or GST fusion proteins (0.5 μg) in 100 μL were attached to a glutathione plate (BD Biosciences, USA) by incubation at room temperature for 1 hr, followed by incubation with 5% (w/v) skim milk in PBS-T overnight at 4 °C. After washing the plate six times with PBS-T, the plate was incubated with FITC-labeled Gα protein (100 μL/well) in buffer B for 1 hr at room temperature. The amount of bound FITC-labeled Gα protein was eluted into solution by incubation with 100 μL of 8 M urea solution for 15 min. The intensity of fluorescence in the solution was measured at the excitation wavelength of 495 nm and at the emission wavelength of 525 nm using a TRIAD microplate reader (Dynex Technologies, USA). The fluorescence intensity from each Gα protein bound to immobilized GST was subtracted from the intensity from Gα proteins bound to specific GST-fusion proteins. Alternatively, the interaction between Gαi3 and GST-iL3_{BLT2} was measured by enzyme linked immunosorbent assay (ELISA) using unlabeled Gαi3. After binding GST-iL3_{BLT2} (0.5 μg) onto a glutathione plate, serially diluted Gαi3 was added. The plate was incubated (100 μL/well) for 90 min at room temperature, and washed 6 times (300 μL/well) with PBS-T to remove unbound proteins. The complex was incubated for 1 hr with 100 μL of anti-His tag antibody (1:3000, Sigma) and for 1 hr with HRP-conjugated anti-rabbit IgG (1:3000, Sigma). The plate was washed 6-times with PBS-T, and 100 ml of OPD (1 mg/mL) in 1X stable peroxide substrate buffer (Pierce) was added. The reaction was terminated by the addition of 100 μL of 2.5 M sulfuric acid, and the absorbance at 490 nm was measured using a TRIAD microplate reader (Dynex Technologies, USA).

Results

Preparation of GST fusion proteins and various Gα subunits. To characterize the interaction mode of hBLT2 with various Gα proteins, GST-fusion proteins containing the intracellular loop regions of hBLT2 were designed as

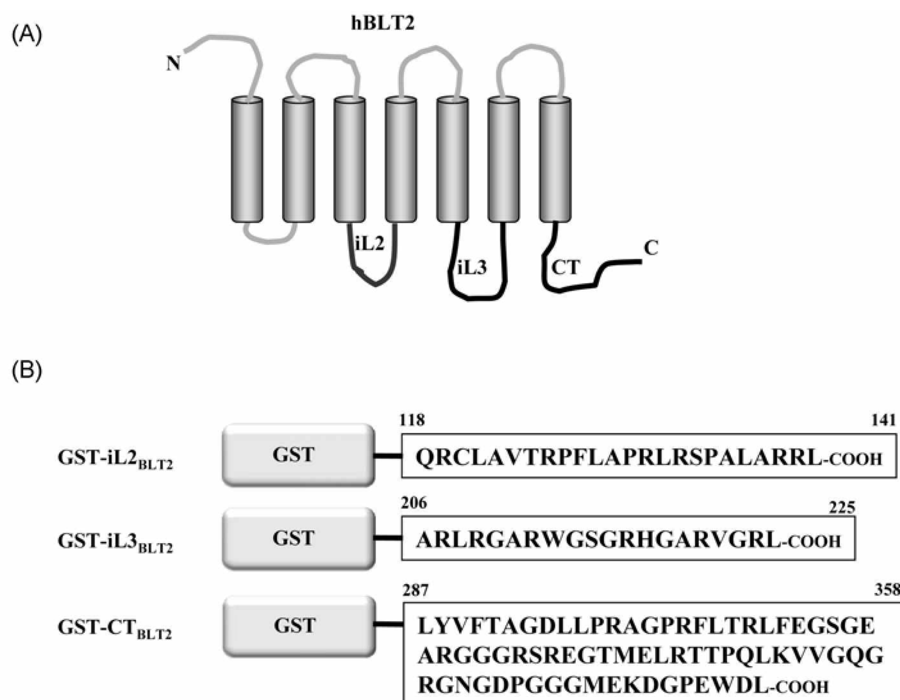

Figure 1. Schematic representation of GST fusion protein of the intracellular domains of hBLT2. (A) The transmembrane helixes of hBLT2 were indicated as cylinders, and iL2, iL3 and CT regions were indicated as thick black lines. along with the residue numbers at the N- and C-terminus of each region. (B) The GST-fusion proteins contained iL2, iL3 and CT at the C-terminus of glutathione S-transferase (GST). The residue numbers of iL2, iL3 and CT are indicated at the N- and C-terminus of each region.

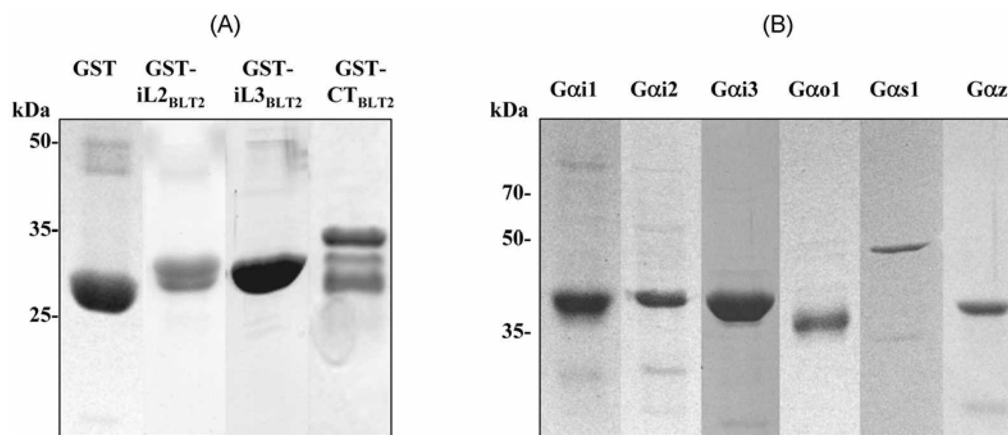

Figure 2. Purification of GST-iL2$_{BLT2}$ GST-iL3$_{BLT2}$, GST-CT$_{BLT2}$ and Gα proteins. (A) SDS-PAGE of purified GST, GST-iL2$_{BLT2}$ GST-iL3$_{BLT2}$, and GST-CT $_{BLT2}$. (B) SDS-PAGE of purified Gα proteins.

depicted in Figure 1, and expression vectors for these GST fusion proteins and various Gα proteins were constructed. The GST-fusion proteins containing the second intracellular loop (iL2). the third (iL3) intracellular loop or the C-terminal tail (CT) of hBLT2 at the C-terminus of GST was also expressed in *E. coli* as soluble proteins. These fusion proteins (GST-iL2$_{BLT2}$. GST-iL3$_{BLT2}$, and GST-CT$_{BLT2}$) were purified semi-homogeneity and the molecular weights of the purified GST-iL2$_{BLT2}$. GST-iL3$_{BLT2}$, and GST-CT$_{BLT2}$ were determined to be 29.4, 30 and 34.4 kDa. respectively (Fig. 2A). The recombinant Gαs1. Gαi1. Gαi2. Gαi3. Gαo1, and Gαz were purified to homogeneity using an ion exchange and Ni^{2+}-affinty column (Fig.2B). Approximately 20 -30% of the bacterially expressed Gαi1. Gαi2 and Gαo1 were recovered as soluble protein (data not shown) and a minimum of 2 mg of purified protein were obtained from 1 L cultures. Most of the expressed Gαi3 was expressed as soluble protein. and 10 mg of purified protein was obtained from 1 L culture. The Gαs1 was purified as described previously.[23] In the case of Gαz, only about 5% was recovered as a soluble fraction and the yield was 0.5 mg of purified protein per 1 L culture. All the purified Gα subunits were labeled with FITC. and the average molar ratio of fluorescence label to Gα proteins was 1.5 to 2.0, based on the fluorescence intensities relative to the protein concentrations.

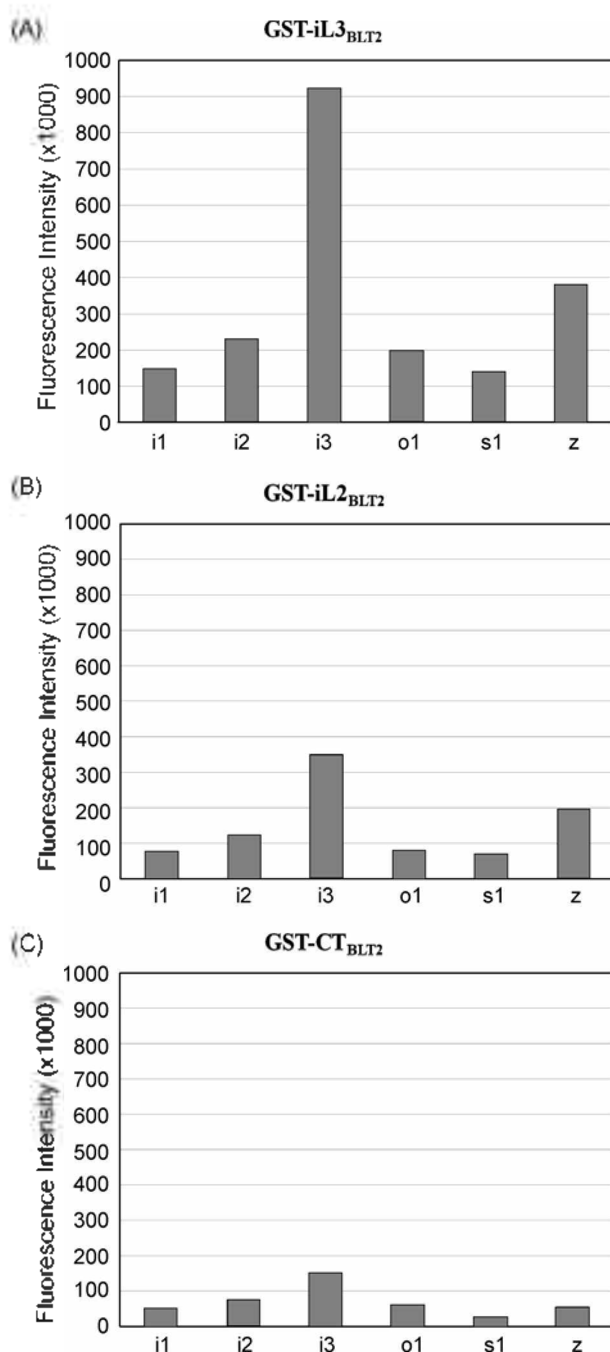

Figure 3. Specific interaction between intracellular regions of hBLT2 and Gα proteins. Purified GST-iL3$_{BLT2}$ (A), GST-iL2$_{BLT2}$ (B), or GST-CT$_{BLT2}$ (C) were immobilized, and the intensities of fluorescence from the bound FITC-labeled Gαi1 (i1), Gαi2 (i2), Gαi3 (i3), Gαo1 (o1), Gαs1 (s1) and Gαz (z) were measured.

The iL3 region of hBLT2 and Gαi3 are major determinants for the interaction of hBLT2 with G-protein. The involvement of Gi has been implicated in the signaling pathway of hBLT2.[16] In order to identify specific G-protein that could bind to hBLT2, the affinity of hBLT2 to various Gα proteins was measured, since the Gα subunit of heterotrimeric G-protein mainly contributes its interactive effect via GPCR.[24-26] Also, the cytoplasmic loop regions of GPCR connecting the transmembrane helices or the C-terminus tail

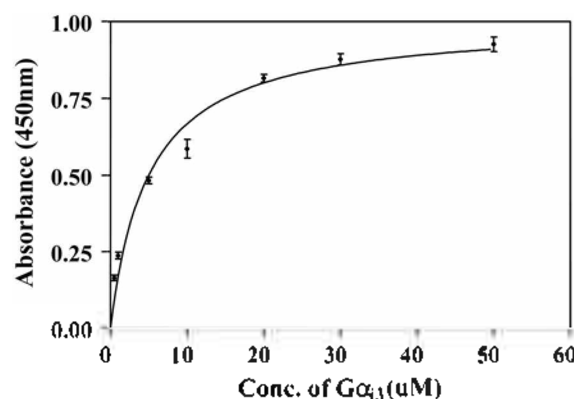

Figure 4. A dose-dependent binding curve of Gαi3 to the immobilized GST-iL3$_{BLT2}$ was measured using anti-His tag antibody, and the K_D value was obtained from a nonlinear regression fitting of the hyperbola function using SigmaPlot version 9.

region could interact with G-proteins. To identify the critical region(s) of hBLT2 for the interaction with Gαi3, the binding affinities of the cytoplasmic regions of hBLT2 to Gαi3 were examined using GST-iL2$_{BLT2}$, GST-iL3$_{BLT2}$ or GST-CT$_{BLT2}$, since these regions have been implicated in the signaling pathway of GPCRs.[23,27] The relative binding affinities of various Gα proteins were compared by measuring the amount of FITC-labeled Gα proteins which bound to the GST fusion proteins. As shown in Figure 3A, GST-iL3$_{BLT2}$ strongly binds to Gαi3. Also Gαz showed marginal binding to GST-iL3$_{BLT2}$. Other tested Gα proteins displayed no significant binding to the GST-iL3$_{BLT2}$. On the contrary, GST-iL2$_{BLT2}$ and GST-CT$_{BLT2}$ could not bind to the Gα proteins (Fig. 3B, C). The binding kinetics of FITC-labeled Gαi3 to GST-iL3$_{BLT2}$ was further examined by measuring the bound amount of Gαi3 at different concentrations, and the apparent dissociation constant (K_D) of FITC-labeled Gαi3 to GST-iL3$_{BLT2}$ was calculated as 5.0 μM (Fig. 4). These results indicate that Gαi3 has the highest binding affinity to hBLT2 among the Gα proteins of the Gi family, and Gi3 might be responsible for the signaling of hBLT2 that induces PT-sensitive chemotactic movement.[16]

Discussion

The characterization of the interaction between hBLT2 and Gα proteins has been reported in this study. Multiple G-proteins had been implicated in the mediation of the signaling of hBLT2. Activation of cells expressing hBLT2 by LTB4 induced an increase of cellular calcium concentration, and a reduction of cAMP as well as the chemotactic response, implying that G-proteins which belong to the Gi or Gq families might interact with hBLT2.[16] These responses were interfered with the treatment of PT. In particular, hBLT2-mediated chemotactic response was completely blocked by PT.[16] Since PT modifies Gα subunits of Gi families, a member of Gi family is assumed to mediate hBLT2-mediated chemotactic response. As shown in Figure 3, Gαi3 showed the highest binding to the intracellular loop

regions of hBLT2 among the three tested Gαi subunits, suggesting that Gαi3 is a potential candidate for mediating the LTB4-dependent chemotactic response in cells expressing hBLT2. The wide distribution of Gi3 and hBLT2 in various types of tissues also supports the involvement of Gi3 in the signaling of hBLT2.[16,28] Also, the importance of the iL3 region of hBLT2 for the interaction with Gαi3 was demonstrated in this study. Involvement of the iL3 region as a major contributor for the interaction with G-protein had been reported in other GPCRs. Mutation in the iL3 region abolished proper signaling of the β-adrenergic receptor[29] or serotonin receptor 5-HT_{2A}.[30] Of note, Gαs1 bound specifically to the iL3 of serotonin receptor type 6, and the single amino acid substitutions impaired signaling efficiency of the receptor.[23] Among the tested GST-fusion proteins, GST-$iL3_{BLT2}$ exhibited strong binding to Gαi3 (Fig. 3), whereas the GST-$iL2_{BLT2}$ and GST-CT_{BLT2} failed to display any detectable association with the Gα subunits, indicating that the iL2 or CT region of hBLT2 might be involved in the signaling process either by interacting with Gβ or Gγ subunits or mediating receptor recycling rather than by direct interaction with Gαi3.

The binding affinity between Gαi3 and GST-$iL3_{BLT2}$ (with K_D value of 5.0×10^{-6} M) was substantially lower than the affinity of the refolded BLT1 for heterotrimeric G-proteins with a K_D value of 7.8×10^{-8} M,[31] suggesting that the affinity of the hBLT2 to the heterotrimeric G-protein would probably be higher than the affinity to the isolated Gαi3 subunit, and the Gβγ subunits may be involved in the interaction with iL3 or other cytoplasmic regions of hBLT2. Further study on the ligand-induced conformational change of hBLT2 and its interaction with the Gi3 protein should reveal the signaling mechanism of hBLT2.

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