A Combinatorial G Protein-coupled Receptor
Reconstitution System on Budded Baculovirus

EVIDENCE FOR Gαi AND Gαo, COUPLING TO A HUMAN LEUKOTRIENE B4 RECEPTOR*

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To investigate the coupling selectivity of G proteins and G protein-coupled receptors (GPCRs), we developed a reconstitution system made up of GPCR and heterotrimeric G proteins on extracellular baculovirus particles (budded virus (BV)). BV released from SF9 cells infected with a recombinant baculovirus coding for human leukotriene B4 receptor (BLT1) cDNA exhibited a high level of BLT1 expression (27.3 pmol/mg of protein) and specific [3H]leukotriene B4 binding activity (Kd = 3.67 nM). The apparent low affinity of the expressed BLT1 is thought to be due to relative non-availability of the Gαi isoform, which couples to BLT1, in BV. Co-infection of heterotrimeric G protein recombinant viruses led to co-expression of BLT1 and G protein subunits on BV. A guanosine-5′-[(βγ)-imidophosphatidate-sensitive, high affinity ligand binding was observed in the BLT1 BV co-expressing Gαi1β1γ2 (Kd = 0.17 nM). A relatively large amount of high affinity receptor protein was recovered in the co-expressing BV fraction (6.81 pmol/mg of protein). A combination of BLT1 and Gαi1 without Gβ1γ2 did not exhibit high affinity ligand binding on BV, indicating the low background environment for the GPCR-G protein coupling in this BV reconstitution system. To test other G proteins for coupling, various Gα subunits were combinatorially expressed in BV with BLT1 and Gβ1γ2. The BLT1 BV co-expressing Gα1a1β1γ2 exhibited a comparably high affinity ligand binding as well as ligand-stimulated guanosine 5′-3-O-(thiol)triphosphate binding to Gα1a1β1γ2. Co-expression of other Gα isoforms such as Gαo, Gα11, Gα14, Gα16, Gα27, or Gα13 did not exhibit any significant effects on ligand binding affinity in this system. These results reveal that BLT1 and coupled trimeric G proteins were functionally reconstituted on BV and that Gαi, as well as Gαo, couples to BLT1. This expression system should prove highly useful for pharmacological characterization, biosensor chip applications, and also drug discovery directed at highly important targets of the membrane receptor proteins.

G protein-coupled receptors (GPCRs) comprise a superfamily of membrane proteins made up of seven transmembrane segments. GPCRs play an important role in transmembrane signal transduction by binding extracellular ligands such as biogenic amines, peptides, hormones, lipids, and nucleotides. The sensing of exogenous stimuli such as light, odors, and taste is also mediated by this family of GPCRs. Ligand binding activates the heterotrimeric guanine nucleotide-binding proteins (G proteins) on the intracellular face of the plasma membrane. There is a considerable interest in the investigation of newly identified GPCRs as potential therapeutic targets. The expression of GPCRs in insect cells with recombinant baculovirus is a common technique. SF9 cells, derived from Spodoptera frugiperda, are essentially free of endogenous GPCRs, providing a low background environment for ligand binding assays. SF9 cells do endogenously contain certain classes of G proteins that couple to heterologously expressed GPCRs. However, various lines of evidence have made it clear the endogenous Gα-class G proteins exist only at low levels in SF9 cells (11, 12). The 5-hydroxytryptamine (10, 13–15), M2 muscarinic (16), D2 dopamine (17), formylmethionylleucylphenylalanine (11) receptors have been shown to increase ligand binding activity and/or GTP binding

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activity in Sf9 cells when co-expressed with G₁₂-class G proteins.

Although Sf9 cells are able to support very high expression levels of exogenous genes (19), the D₂ dopamine receptor expressed in Sf9 cells is largely unglycosylated (20). In the case of the β₂-adrenergic receptor and the formylmethionylleucylphenylalanine receptor, immature and incompletely glycosylated forms of these receptors in the cell membrane fraction of Sf9 cells have also been reported (11, 21, 22).

Loisel et al. (23) successfully recovered functional β₂-adrenergic receptor from extracellular baculovirus particles by co-infection of β₂-adrenergic receptor recombinant baculovirus with a baculovirus encoding the Pr55 Gag product of the human immunodeficiency virus type 1. In contrast with what had been expected, most of the recombinant β₂-adrenergic receptor was excluded from the Gag particles and were found instead to be associated with extracellular baculovirus (BV) in a budded form. Surprisingly, the β₂-adrenergic receptor expressed on the virus particles had been glycosylated and was biologically active. In addition, post-translational modifications were found upon agonist stimulation. It should be noted here that β₂-adrenergic receptor is a Gα₁₁-coupling receptor (24), and Sf9 cells contain the Gα₁₁ class G protein in some abundance (12).

BLT1 is known to be the high affinity receptor for leukotriene B₄ (LTB₄), which is found mainly in leukocytes and has been implicated in inflammatory activity (25–28). BLT1 is also reported to couple to Gi- and Gq-class G proteins (29, 30) and is not involved in Gα₁₁-class G protein signaling. In a previous report, guinea pig brain membrane exhibited LTB₄-specific binding (31). It is also reported that LTB₄ binding to partially purified membrane fraction from porcine leukocytes was in-

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**FIG. 1. Time course of BLT1 expression on budded virus and Sf9 cells.** The expression of His-tagged BLT1 was detected by ELISA with anti-His antibody (a) or by [³H]LTB₄ binding (b). The BV and membrane fractions were collected at various times after infection. a, the BV fractions were collected from Sf9 cells infected with His-BLT1 recombinant (●) or wild type (wt, ■) baculovirus. The membrane fractions were also prepared from Sf9 cells infected with His-BLT1 recombinant (○) or wild type virus (□). Samples (5 μg of protein) were used to detect the immunoreactivity. b, 1 μg of protein of the BV or membrane fractions was incubated with 0.25 nM [³H]LTB₄, and bound radioactivity was detected. Data represent the mean ± S.D. (n = 4).
creased by reconstitution of G\(_i\)-class G proteins including G\(_i\)/H9251, which is found in the brain and nervous system (33). Although these reports suggest that G\(_i\)/H9251 mediates LTB\(_4\) signaling, the coupling of G\(_i\)/H9251 with BLT1 has not been investigated intensively. To exploit extracellular baculovirus expression to test whether GPCR and G protein could be reconstituted on the BV, we studied the expression of human BLT1. The possible selectivity of BLT1 coupling with other classes of G proteins was also studied by means of this system.

**EXPERIMENTAL PROCEDURES**

Reagents—LTB\(_4\) was purchased from Cayman Chemical Co. (Ann Arbor, MI). [\(^{3}H\)]LTB\(_4\) (6016.2 GBq/mmol) and [\(^{35}S\)]GTP\(_\gamma\)S (46.2 TBq/mmol) were purchased from PerkinElmer Life Sciences. Other chemicals or reagents were purchased from Sigma unless otherwise noted. A recombinant baculovirus expression vector pAcYMI (34) was kindly provided by Dr. Y. Matsuura (the Research Institute for Microbial Disease, Osaka University, Japan). A baculovirus-expressing bovine G\(_i\)/H9252 (35) was generously provided by Dr. T. Kozasa (the Department of Pharmacology, University of Illinois at Chicago). The cDNA clones for human G\(_i\)/H9251, G\(_i\)/H9251, G\(_i\)/H9251, and G\(_i\)/H9251 were provided by the Guthrie cDNA Resource Center (www.cdna.org).

Recombinant Baculovirus Construction and Sf9 Cell Culture—The cDNA for human BLT1 (29) was amplified by PCR using a 5’ sense primer containing the BamHI site and a 3’ antisense primer containing the EcoRI site and inserted into pBlueBac4.5 and pBlueBacHis2A bacu-
The membrane fraction and BV fraction were prepared from infected His-BLT1 and G primer containing the Sma site at the type (wt and treated with Klenow fragment to generate blunt ends. Baculovirus and G Sma supplemented media (In-}

The recombinant baculoviruses thus obtained were plaque-purified and treated with Klenow fragment to generate blunt ends. Baculovirus and G Sma supplemented media (In-

All viruses were produced by homologous recombination in Sf9 cells. The recombinant baculoviruses thus obtained were plaque-purified and amplified. Sf9 cells were cultured in Grace’s supplemented media (Invitrogen) containing 10% fetal calf serum, 0.1% Phoronic F-68 (Invitro-

Reconstitution of GPCR and Heterotrimeric G Proteins

Expression levels of BLT1 on BV co-expressing G protein subunits. BV samples expressing His-tagged BLT1 with or without various G protein subunits were subjected to ELISA with an anti-His antibody (n = 4, mean ± S.D.). Samples of 3 μg of protein were used. β2 and wild type (wt) represent the BV expressing Gβ2 plus Gγ2 subunits and the budded wild type baculovirus, respectively. OD, optical density.

Sucrose Density Gradient Sedimentation—Fractionation of the BV fraction by sucrose density gradient sedimentation was performed as described by Loisel et al. (23). Briefly, 600 μg of the BV fraction, which had been suspended in 1.2 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was layered onto 36 ml of a linear 25–70% sucrose gradient in TE buffer. The sample was centrifuged at 10,000 g for 90 min. Fractions of 1.5 ml were collected from the top of the tube. Then each fraction was centrifuged at 50,000 × g for 45 min. The precipitates were resuspended with phosphate-buffered saline and used for Western blot analysis.

Electrophoresis and Immunoblotting—SDS-PAGE was carried out as described by Laemmli (41), except that samples were not heat-treated so as to minimize aggregation. For immunoblotting, proteins expressed on BV were resolved by SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences) electrophoretically at 20 V overnight. Membranes were incubated for 1 h with Block Ace (Snow Brand Milk Products, Japan) at room temperature. Membranes were then incubated overnight at 4 °C with the primary antibody in Tris-buffered saline (TBS; 137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.4). The following antibodies were used: monoclonal mouse anti-baculo-

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goat anti-Ga13 (C-18 sc-7415) (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal rabbit anti-Ga11 (AS/7, PerkinElmer Life Sciences); anti-Ga13 (3A-190, Gramsch Laboratories, Schwabhausen, Germany); anti-Ga14 (3A-195, Gramsch Laboratories). Membranes were washed 4 times with TBS with 0.05% Tween 20 and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG as the secondary antibody. Membranes were then washed 3 times with TBS with 0.05% Tween 20 and incubated with SuperSignal West Dura substrate (Pierce). Membranes were then exposed to x-ray film RX-U (Fuji Photo Film, Tokyo, Japan).

**Enzyme-linked Immunosorbent Assay**—The BV or membrane fractions were adsorbed to the wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Asahi Techno Glass, Tokyo, Japan) overnight at 4 °C. Wells were washed and blocked with TBS, pH 7.4, containing 40% Block Ace for 1 h at room temperature. After incubation with mouse monoclonal anti-polyhistidine antibody for at least 1 h, wells were washed 3 times with TBS with 0.05% Tween 20. The anti-polyhistidine antibody bound to wells was detected with the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody followed by the addition of substrate (tetramethylbenzidine). The reaction was terminated by acid, and absorbances at 450 nm were quantitated using a 96-well plate reader.

**Ligand Binding Assay**—The radiolabeled ligand binding assay was carried out in a total of 200 μl of binding buffer (50 mM Tris-HCl pH 7.4, 10 mM MgCl2, 10 mM NaCl, 0.1% fatty acid-free bovine serum albumin) containing the BV fraction and [3H]LTB4. To test the effect of Gpp(NH)p on [3H]LTB4 binding to the BV fractions, 0.02% (w/v) of saponin was added in the binding buffer with various amounts of Gpp(NH)p. The assay mixtures were incubated at room temperature for 1 h. The incubation was terminated by filtration of the assay mix-
Reconstitution of GPCR and Heterotrimeric G Proteins

Expression of BLT1 on Budded Baculovirus

The BV fraction was recovered from the culture medium of SF9 cells infected with His-tagged BLT1 (His-BLT1) recombinant baculovirus. BLT1 expression, detected by ELISA with anti-His antibody, was seen in the BV fraction 48 h after infection with recombinant virus carrying His-BLT1 cDNA (Fig. 1a). The His-BLT1 expression reached its peak 72 h after infection. The amount of His-BLT1 in the BV fraction at 72 h was 1.4-fold higher than that of the membrane fraction from His-BLT1-infected SF9 cells. [3H]LTB4 binding activity was also observed in both the BV and membrane fractions prepared from SF9 cells that had been infected with His-BLT1 recombinant baculovirus (Fig. 1b). The maximum binding activity was reached at 72 h after infection, which was about 3-fold higher in the BV than the membrane fraction. No binding activity was detected in the BV or membrane fraction collected from SF9 cells infected with wild type baculovirus.

To clarify whether BLT1 is expressed on virus or cell debris, the BV fraction from the culture medium of SF9 cells infected with BLT1 recombinant baculovirus was analyzed by ultracentrifugation in sucrose density gradients. As shown in Fig. 2, His-BLT1 was found to coexist with baculovirus glycoprotein gp64. The expression time course and properties of the sedimentation of BLT1 in culture supernatant are compatible with previously reported β2-adrenergic receptor expression on extracellular budded virus (23). Thus, BLT1 is likewise induced in this case to be expressed on the budded baculovirus. The affinity for the [3H]LTB4 of BLT1 expressed on BV seems to be low (Kd = 3.67 nM) (Table I) compared with that of the membrane fraction of Cos-7 cells expressing BLT1 (Kd = 0.17 nM) (29). This is considered to be due to the low level of the Gα-class G protein in SF9 cells, which is needed to couple to BLT1 for high affinity binding.

Reconstitution of BLT1-G Protein Coupling on Budded Virus

Expression of Receptor and Heterotrimeric G Protein—To check for BLT1-G protein coupling on BV, we prepared recombinant baculoviruses containing the cDNAs encoding the His-BLT1 as well as the G protein α1i, β1i, and γ2i subunits. His-BLT1 and the G protein subunits Go1i, GB1i, and Gγ2i were expressed on BV in various combinations. After 72 h of infection, each BV fraction was collected. The amount of expressed receptor or each subunit of G protein was assessed by Western blotting (Fig. 3). When the sample was heat-treated for SDS-PAGE, BLT1 stacked in the stacking gel and did not enter the separating gel. Therefore, we performed SDS-PAGE analysis without heat treatment. For BLT1, the deduced molecular mass from the amino acid sequence is 37.6 kDa. Using anti-His antibody immunoblotting, three bands, with apparent molecular masses of 36.6, 35.5, and 34.8 kDa, were detected in His-BLT1-expressing BV. We did not observe the bands corresponding to the dimmers of the BLT1 in this system even in the presence of G protein. The amount of receptor expression decreased with co-infection of various G protein subunits in both the membrane and BV fractions (Fig. 3a). This observation was corroborated with ELISA measurement using an anti-His antibody (Fig. 4). No immunoreactive band was detected with anti-His antibody in the wild type BV sample. Each G protein subunit expressed an apparently similar amount on BV.

Ligand Binding Assay of Reconstituted Receptor and G Protein—The BV fractions recovered from culture medium 72 h after infection with His-BLT1 recombinant virus displayed a dose-dependent binding for [3H]LTB4 (Fig. 5, a and b). In Scatchard plot analysis, the binding properties of [3H]LTB4 to BLT1 expressed on BV or membrane fractions contain both high and low affinity sites (Fig. 5, c and d). The Ki and Bmax values calculated from the Scatchard plot are shown in Table I. As shown in Table I, the ligand binding properties were similar in the SF9 membrane and BV fractions expressing the receptor alone. For BV-expressed BLT1, the mean Ki and Bmax values of the high affinity binding sites were 0.13 nM and 6.4 pmol/mg of protein, and those of the low affinity sites were 3.67 nM and 27.3 pmol/mg of protein, respectively (n = 4). The His hexapeptide fused to the N terminus of the BLT1 did not affect ligand binding (data not shown). There was no significant specific [3H]LTB4 binding in the BV fraction prepared from the culture medium of SF9 cells infected with the wild type baculovirus.

To estimate the effects of G protein on agonist affinity, the SF9 membrane and BV fractions co-expressing receptor and G proteins were used for the [3H]LTB4 binding experiments (Fig. 5). As shown in Table I, the Bmax value of the membrane fraction co-expressing with BLT1 and Go1i was 3.78 pmol/mg of protein, which is 2.8-fold greater than the membrane fraction expressing BLT1 alone. The co-expression of Gβ1γ2i subunits with BLT1 also increased the high affinity site by 1.5-fold in the membrane fraction. For the BV fraction, the sample co-expressing BLT1 and Go1i exhibited a relatively small number of high affinity binding sites (Bmax = 1.92 pmol/mg of protein). Reconstitution of the G protein heterotrimer (Go1iβ1γ2i) switched almost all the entire receptor population to the high affinity state with a Ki value of 0.17 nM, increasing the Bmax value of the high affinity receptor 9.5-fold compared with the receptor expressed alone.

RESULTS

Expression of BLT1 on Budded Baculovirus

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Fig. 6. The effect of Gpp(NH)p on ligand binding to BLT1 co-expressed with G protein on BV. Specific binding of [3H]LTB4 (added at a concentration of 0.25 nM) to 3 μg of BV (n = 4, mean ± S.D.). Budded virus expressing BLT1 and Go1i, BLT1 and Gα1i, BLT1 and Gβ1γ2i, and BLT1 alone (as indicated in the figure) were analyzed. Statistical analysis was performed using Student’s t test. Significant inhibition by Gpp(NH)p is indicated as p < 0.001 (*).
The Effect of Gpp(NH)p on Ligand Binding to BLT1 and G Proteins

A GTP analogue, Gpp(NH)p, which is not hydrolyzed by the GTPase activity of the Go protein, was used to delineate receptor-G protein coupling. Gpp(NH)p decreased [3H]LTB4 binding to BLT1 and Gβγ2, providing evidence for the receptor-G protein coupling (Fig. 6). On the other hand, the BLT1 BV, BLT1 with Go11 BV, and BLT1 with Go12 BV were not significantly affected in ligand binding by the addition of Gpp(NH)p. These results suggest that the co-expression of the receptor and heterotrimeric G protein reconstitute to functional GPCR-G protein complex on BV.

The Effect of the Co-expression of Various Classes of G Protein on the [3H]Leukotriene B4 Binding Properties of BLT1 Expressed on BV

To compare the effect of the G protein α subunit of various classes on ligand binding, Sf9 cells were co-infected with various combinations of recombinant baculovirus encoding several different classes of G protein subunits (α with Gβγ in every case) and His-BLT1. Each BV fraction was collected by centrifugation, and each of the expressed proteins was detected by Western blotting (Fig. 7). The expression levels of His-BLT1 assessed by ELISA were similar among the BV fractions expressing different G proteins (data not shown). No specific
Fig. 8. [3H]LTB4 binding to the BV fractions co-expressing BLT1 and various classes of Ga with Gβγ. Binding isotherms of BV co-expressing BLT1 and various classes of Ga subunits with Gβγ are shown (n = 4, mean ± S.E.). [3H]LTB4 binding to budded viruses expressing BLT1 and Gaβγ (□), BLT1 and Gaγ (○), BLT1 and Gaβγ (△), BLT1 and Gaα (△), BLT1 and Gaβγ (△), BLT1 and Gaα (△), BLT1 and Gaβγ (△), BLT1 and Gα (△), and BLT1 alone (+) were analyzed. In each assay point, 1 μg of BV expressing BLT1 alone or 2 μg of that of co-expressing BLT1 with Gaβγ, Gaα, or Gαβγ was used. For other BV samples, 3 μg were used. Isotherms were best fitted to a two-binding-site model using nonlinear analysis. The $K_d$ and $B_{\text{max}}$ values were derived for each individual experiment, and means ± S.E. are summarized in Table II.

| Coexpressed proteins | High affinity binding | Low affinity binding |
|----------------------|-----------------------|---------------------|
|                      | $K_d$ (mean ± S.E.) nm | $B_{\text{max}}$ (mean ± S.E.) pmol | $K_d$ (mean ± S.E.) nm | $B_{\text{max}}$ (mean ± S.E.) pmol |
| BLT1 Gaβγ           | 0.174 ± 0.057         | 0.476 ± 0.083       | 3.437 ± 0.178         | 4.716 ± 0.311 |
| BLT1 Gaαβγ          | 0.171 ± 0.014         | 5.139 ± 0.133       | ND                    | ND               |
| BLT1 Gaβγ           | 0.334 ± 0.057         | 5.141 ± 0.317       | ND                    | ND               |
| BLT1 Gαβγ           | 0.144 ± 0.049         | 0.340 ± 0.057       | 3.490 ± 0.118         | 5.278 ± 0.228 |
| BLT1 Gaαβγ          | 0.109 ± 0.045         | 0.396 ± 0.064       | 3.582 ± 0.149         | 5.587 ± 0.275 |
| BLT1 Gaαβγ          | 0.121 ± 0.060         | 0.200 ± 0.047       | 3.523 ± 0.078         | 7.055 ± 0.193 |
| BLT1 Gaαβγ          | 0.199 ± 0.177         | 0.163 ± 0.078       | 3.715 ± 0.137         | 6.374 ± 0.302 |
| BLT1 Gaαβγ          | 0.165 ± 0.068         | 0.061 ± 0.023       | 3.505 ± 0.078         | 5.720 ± 0.090 |
| BLT1 Gaαβγ          | 0.146 ± 0.944         | 0.038 ± 0.067       | 3.498 ± 0.058         | 22.82 ± 0.267 |

signals were detected by Western blotting in the BV fraction recovered from the culture medium of Sf9 cells infected with wild type baculovirus. The ligand binding properties of these BV fractions were compared. As shown in Fig. 8, an increase of high affinity binding sites was observed in the BV co-infected with His-BLT1 and the Gα heterotrimeric G protein (Gααβγ). Furthermore, co-expression of Gα heterotrimeric G protein with His-BLT1 (Gααβγ) also led to an increase in the high affinity binding sites. On the other hand, BV fractions co-expressing His-BLT1 with other classes of G protein heterotrimers (Gαα, Gαγ, Gαβα, Gαβγ, and Gγγ) exhibited a low affinity ligand binding similar to that of BV expressing His-BLT1 alone. As shown in Table II, the $B_{\text{max}}$ value of BV co-expressing His-BLT1 and Gααβγ or Gααβγ in the case of the high affinity sites was similar to that of BV co-expressing other classes of heterotrimeric G proteins in the low affinity sites. These results indicate that almost all the expressed receptors in the former two BV fractions were functionally coupled to Gααβγ and Gααβγ.

[3S]GTPγS Binding of Heterotrimeric G Proteins Expressed in Budded Virus

To further examine the selective coupling of G proteins to BLT1, the displacement of [3S]GTPγS binding by GDP in the presence or absence of 100 nM LTB4 was compared among the various BLT1 BV co-expressing different combinations of heterotrimeric G proteins (Fig. 9). Previous studies demonstrate that the agonist increases the apparent affinity of [3S]GTPγS binding sites; it also decreases the affinity of GDP binding sites (44, 45). The binding of [3S]GTPγS was greatly increased by LTB4 in BV co-expressing BLT1 and Gα or Gγ heterotrimeric G proteins. Displacement curves for BLT1 BV co-expressed with Gααβγ or Gααβγ were similar to each other both in the presence or absence of the ligand (Fig. 9, b and c). The IC50 values for the GDP of BLT1 BV co-expressing Gααβγ or Gααβγ were 0.25 and 0.80 μM in the absence of LTB4, and 7.58 and 12.45 μM in the presence of LTB4, respectively. These results show that BLT1 couples to Gα, as well as Gγ heterotrimeric G proteins in BV.

For the BLT1 BV co-expressing Gα-class G proteins, LTB4 did not exhibit a significant effect on the displacement of [3S]GTPγS binding by GDP (Fig. 9a). Although the binding of [3S]GTPγS was apparently induced by LTB4 in BLT1 BV co-expressed with Gα-class heterotrimeric G proteins, the effects of LTB4 were much less than that for BLT1 BV co-expressed with Gα-class G proteins (Fig. 9, d-f). The IC50 values for [3S]GTPγS binding on BLT1 BV co-expressed with Gα-class G proteins (Gααβγ, Gααβγ, and Gααβγ) were increased by 2.3-, 2.4-, and 3.1-fold, respectively, in the presence of LTB4. The BLT1 BV co-expressing Gγβγ-class G protein also showed a slight difference in [3S]GTPγS binding by LTB4 (Fig. 9, g and h). The IC50 values for the GDP of BLT1 BV co-expressed with Gααβγ and Gααβγ were increased by 2.3- and 2.6-fold, respectively, in the presence of LTB4.

DISCUSSION

Baculovirus infection of Sf9 cells renders the recombinant membrane proteins functionally embedded in the viral parti-
cles (23). We further exploited this feature by using a co-expression system with GPCR and G proteins to reconstitute the high affinity receptor. In addition, we observed that the combinatorial co-expression of individual G protein subunits could accommodate GPCR-G protein coupling specificity in terms of ligand affinity.

Sf9 cells infected with His-BLT1 baculovirus released BV on which the BLT1 receptor was expressed. The BV fraction exhibited higher ligand binding activity than the membrane fraction, whereas the membrane fraction contained an excess amount of receptor protein. These results are in good accordance with a previous report that addressed β2-adrenergic receptor expression on extracellular virus (23). Human β2-adrenergic receptor expressed on the BV of baculovirus exhibited specific ligand binding activity (23). β2-Adrenergic receptor is known to couple to the Gs class of G protein (24). Because Gs is abundantly present in Sf9 cells, the recovered baculovirus contained sufficient intrinsic Gs-class G protein such that evidence provided that the functional high affinity receptor was expressed in BV (23).

When BLT1 was expressed alone, the BV fraction exhibited a small number of high affinity and a large number of low affinity ligand binding sites. The $K_d$ value of LTB4 to BLT1 expressed on BV in the high affinity state was 0.126 nM, which is similar to that of human BLT1 expressed in Cos-7 cells previously reported (29). The high affinity and GTP-sensitive binding sites are thought to reflect receptors coupled to G proteins, whereas the low affinity sites represent uncoupled receptors. In Sf9 cells, various G protein subtypes are expressed in different amounts depending on the class of G pro-

**FIG. 9.** Effect of LTB4 on $[^{35}S]$GTPγS binding to various classes of Gα subunit expressed on BV with BLT1 and Gβγ, BV co-expressing BLT1 and various classes of Gα subunit with Gβγ, were incubated with 0.3 nM $[^{35}S]$GTPγS plus 0.1 nM to 0.1 mM of GDP in the presence (■) and absence (□) of 100 nM LTB4. Displacement of $[^{35}S]$GTPγS binding by GDP is shown ($n = 4$, mean ± S.E.). WT, wild type.
protein (12, 46). The small number of the high affinity binding sites observed in BV expressing BLT1 alone may represent a receptor population coupled to endogenous G proteins.

In this study, we co-infected Sf9 cells with recombinant baculoviruses containing the cDNAs for BLT1 and mammalian heterotrimeric G protein (Gα1,β1γ2). The Gβ1γ2 combination used here is capable of interacting with most classes of the G protein α subunit (13, 47). In each experiment, the expression of the receptor and G protein subunits was confirmed with immunoblotting (Fig. 3). The expression level of each component in recovered BV correlated well to the amount of recombinant baculovirus used to infect cells. The majority of the receptors expressed in the membrane fraction from Sf9 cells co-infected with BLT1 and Gα1,β1γ2 recombinant baculoviruses exhibited high affinity ligand binding (Fig. 5, a and c). In the membrane fraction, co-expression of BLT1 and Gα1,β1γ2 increased high affinity ligand binding sites 3.6-fold compared with BLT1 alone. Even co-expression of BLT1 with Gα1 alone led to a 2.8-fold increase in the high affinity binding sites compared with that of BLT1 by itself (Fig. 5c, Table I). Because Gβ subunits have been reported to be detected in Sf9 cells (46), the endogenous Gβγ subunits of Sf9 cells could well be re- cruited to the membrane fraction co-expressing receptor and the Gα subunit and could reconstitute the heterotrimer with exogenous Gαi1. The BV released from the co-infected cells also exhibited high affinity for ligand binding comparable with the binding affinity observed in mammalian expression systems (29) (Fig. 5, b and d). For the BV fraction, co-expression of BLT1 and Gα1 without Gβγ resulted in only a slight increase in the high affinity binding sites (Fig. 5, b and d), probably because BV contains a smaller amount of the endogenous Gβγ subunits. It was necessary for BV to express both Gα1 and Gβγ together with BLT1 to achieve maximum binding (Fig. 5d, Table I). These results are compatible with the finding that Gβγ complex is required for optimal receptor-G protein interaction (13, 17, 18, 47). The changes in the number of high affinity ligand binding sites on BV depend on the expression of exogenous G protein subunits. From these results, a BV co-expression system could provide a better method for the reconstitution of GPCR and G proteins because the background of the endogenous G proteins is lower than in the cell membrane fraction.

The Sf9 cell expression system has also been used to study the coupling specificity of receptors toward their cognate G proteins (14, 17). Interestingly, BLT1 BV co-expressed with Gα1,β1γ2 as well as Gα1,β1γ2 exhibited high affinity ligand binding (Fig. 8) and also ligand-induced GTPγS binding (Fig. 9). These data indicate that Gα1,β1γ2 is able to couple to BLT1 and that ligand-induced activation of the receptor led Gα1 to become a GTP-bound form. Consistent with our findings, a previous report also showed that partially purified LTB4 receptor from porcine leukocytes was able to couple with exogenous Gα1,β1γ2 class G proteins, including Goα (32). The physiological relevance of the BLT1 signaling pathway mediating Goα remains to be elucidated. Goα is reported to be expressed only in brain and neuronal tissues (33) and mediates neurotransmitter receptor signaling. Human BLT1 mRNA expresses mainly in peripheral leukocytes and also in spleen and thymus, albeit at low levels (29). Recently, BLT1 expression was reported in C6 astrogloma cells (48). A specific antagonist for BLT1 markedly inhibited β-amyloid-induced generation of reactive oxygen in C6 cells (48). BLT1 expression was also detected in the hippocampus of brain and other tissues in a GeneChip analysis of normal human tissues (System Biology and Medicine Data base, LSBM, RCAST, The University of Tokyo, www2.genome.rcast.u-tokyo.ac.jp data base). These observations are suggestive of a physiological function for BLT1 in the central nervous system.

The co-expression of BLT1 with any one of the other G proteins tested (Gα1i, Gα14i, Gα16i, Gα12i, and Gα13 with Gβ1γ2 in every case) had no significant effect on ligand binding (Fig. 8). A previous report showed the Gα1 did not couple to BLT1 in the cAMP response (29). The Gα14, but not Gα12, was considered to be coupled to BLT1 in inositol phosphate production (30). For the BLT1 BV co-expressing heterotrimeric Gαi1 class G protein, we observed a slight difference in the displacement of [35S]GTPγS binding by GDP in the presence or absence of ligand (Fig. 9, d-f). The rank order of ligand-induced [35S]GTPγS binding was Gα14i > Gα13i > Gα11i, which was comparable with the previous report (30). For the Gα12i class G protein, it is speculated that Gα13 also mediates BLT1 signaling functions such as chemotactic attraction of peripheral leukocytes. Although Gα12 and Gα13 belong to the same class of G proteins, they mediate different signals and generate overlapping effects (5). Gα13i has been reported to directly interact with and activate p115 RhodGF, a guanine nucleotide exchange factor for the GTPase Rh, which activates Rho signaling (49, 50). Gα13-mediated Rho signaling regulates a variety of cellular responses such as chemotaxis, cell-cycle progression, and axonal guidance by controlling the organization of the actin cytoskeleton (51). In our findings presented here, BLT1 BV co-expressing Gα13i rather than Gα12i, with Gβ1γ2 exhibited slightly increased [35S]GTPγS binding by LTB4 (Fig. 9, g and h). Taken together, our data demonstrate that BLT1 expressed on BV conserve the specificity of receptor-G protein coupling seen under physiological conditions. This strongly suggests this expression system should prove highly useful for the combinatorial assessment of coupling specificity of newly identified receptors to various classes of Gα subunits.

Recently, the chemokine receptors CCR5 and CXCR4 were reported to have incorporated into murine leukemia virus particles. Murine leukemia virus particles containing chemokine receptors were attached to an optical biosensor and were utilized in antibody and human immunodeficiency virus-1 gp120 binding studies (52). Furthermore, it was possible to immobilize the baculovirus BV on a matrix slide with a density of ~1000 spots/cm², a technological advance that led to the development of the virus chip. In a baculovirus expression system, the GPCR expressed on BV remain active for at least several months at 4 °C (data not shown), except in those rare cases of degradative inactivation. This system is considered to have several advantages over the other available expression systems in terms of application of a biochip sensor. These advantages include the availability of recombinant viruses encoding various G protein subclasses, the reconstitution of high affinity receptors by co-infection of each recombinant virus, as demonstrated here, and the low background of the intrinsic GPCR in S9 cells. This system is also expected to be of considerable utility in efforts to characterize the interaction of GPCR and G proteins as well as other molecules, such as the regulator of G protein signaling.

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A Combinatorial G Protein-coupled Receptor Reconstitution System on Budded Baculovirus: EVIDENCE FOR G αi AND Gαo COUPLING TO A HUMAN LEUKOTRIENE B4 RECEPTOR

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