Phosphorylation-independent β-Arrestin Translocation and Internalization of Leukotriene B₄ Receptors

Venkatakrishna R. Jala‡, Wen-Hai Shao‡, and Bodduluri Haribabu‡§

From the ‡James Graham Brown Cancer Center and The Department of Microbiology & Immunology, University of Louisville Health Sciences Center, Louisville, Kentucky 40202

Leukotriene B₄ (LTB₄) activates the G-protein-coupled receptor leukotriene B₄ receptor 1 (BLT1) to mediate a diverse array of cellular responses in leukocytes including chemotaxis, calcium mobilization, degranulation, and gene expression. To determine the role of phosphorylation in BLT1 regulation, we generated mutants of BLT1 in which all of the serine/threonine residues in the C-tail are converted to alanine or to aspartate/glutamate. These mutants expressed in rat basophilic leukemia RBL-2H3 cells bound LTB₄ with similar affinity and activated all of the known functional activities of BLT1, albeit at different levels. The conversion of phosphorylation sites to alanine resulted in enhanced G-protein-mediated activities, whereas conversion to aspartate/glutamate resulted in reduced responses and a right shift in dose response, indicating that receptor phosphorylation is a critical regulator of G-protein-mediated pathways. Surprisingly, translocation of β-arrestin and receptor internalization was completely independent of BLT1 phosphorylation. Real-time analysis of β-arrestin translocation and receptor internalization using digital fluorescence video microscopy in cells expressing a red fluorescent protein labeled BLT1 and a green fluorescent protein-tagged β-arrestin confirmed phosphorylation-independent β-arrestin translocation and internalization of BLT1. In β-arrestin-deficient mouse embryo fibroblasts, the BLT1 receptors failed to display endosomal localization upon stimulation. In these cells, co-expression of β-arrestin-green fluorescent protein with BLT1-red fluorescent protein resulted in co-localization of BLT1 and β-arrestin upon activation. Thus, receptor phosphorylation-dependent mechanisms regulate G-protein-mediated pathways; however, phosphorylation-independent mechanisms regulate β-arrestin association and internalization of BLT1.

Leukotriene B₄ (LTB₄),¹ a lipid mediator derived from arachidonic acid metabolism, is a potent chemoattractant for neutrophils (1). LTB₄ also activates a wide range of other biological responses in leukocytes including the respiratory burst, granule release, and changes in gene expression (2–4). Two distinct G-protein-coupled receptors, BLT1 and BLT2, were identified from several mammalian sources (3, 5). Whereas the high affinity receptor BLT1 is expressed mainly in phagocytic leukocytes and T-lymphocytes, the low affinity receptor BLT2 is more ubiquitously expressed. A number of studies using gene-targeted mice and antagonists and genetic analysis in mice and humans have implicated the importance of leukotrienes and their receptors in inflammatory diseases such as asthma, rheumatoid arthritis, and atherosclerosis (6, 7). However, the molecular mechanisms of signaling and regulation of leukotriene receptor functions largely remain unexplored.

The activation of BLT1 in primary leukocytes or in cell lines expressing BLT1 results in G-protein activation, phosphoinositide hydrolysis, calcium mobilization, exocytosis, and chemotaxis (3, 8). Although BLT1 couples to both Gᵢ and G_q family of proteins, the activation of Gᵢ is essential for chemotaxis. An eighth helix in the cytoplasmic tail of BLT1 as well as threonine 308 within this region was shown to modulate the receptor activity (9–11). Phosphorylation of GPCRs by a G-protein-coupled receptor kinase results in uncoupling of the receptor from G-proteins (12). This desensitization process is further facilitated by the association of the phosphorylated receptors with the cytoplasmic adaptor, β-arrestin (13). The receptor-β-arrestin complex associates with clathrin and accessory proteins involved in the formation of clathrin-coated pits, ultimately leading to receptor internalization (14, 15). To investigate the role of receptor phosphorylation in BLT1 regulation, we generated a phosphorylation-deficient mutant by conversion of all of the serine/threonine residues on the cytoplasmic tail of BLT1 to alanine. These serine/threonine residues were also mutated to aspartate/glutamate, respectively, to mimic constitutive phosphorylation. Such phosphomimic mutants have been used extensively to simulate constitutive phosphorylation and activation of protein kinases (16). The results showed that phosphorylation of BLT1 is an important regulator of signaling through G-proteins but revealed an unexpected dissociation of receptor phosphorylation from interaction with β-arrestin and internalization.

EXPERIMENTAL PROCEDURES

Materials—[³²P]Orthophosphate (8500–9120 Ci/mmol) and [³H]leukotriene B₄ (163 Ci/mmol) were purchased from PerkinElmer Life Sciences and Amersham Biosciences, respectively. LTB₄ was obtained from Cayman Chemicals. Rat collagen was obtained from Collaborative Biochemicals, and 25 × 80-mm, 8-μm pore size polycarbonate filters were from Neuroprobe Inc. The staining kit (PROTOCOL) was obtained from Fisher. Monoclonal 12CA5 antibody and protein G-agarose were FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MEF, mouse embryonic fibroblast.

¹ The abbreviations used are: LTB₄, leukotriene B₄; GPCR, G-protein-coupled receptor; MEF, mouse embryonic fibroblast; RFP, red fluorescence protein; GFP, green fluorescence protein; BLT1, leukotriene B₄ receptor 1; hBLT1, human leukotriene B₄ receptor 1; WT, wild type;

Received for publication, August 26, 2004, and in revised form, November 10, 2004 Published, JBC Papers in Press, November 23, 2004, DOI 10.1074/jbc.M409821200

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
activity bound was determined in the presence of 1 μM unlabeled ligand. Calcium Measurements—Calcium mobilization was monitored in Indo-I-loaded cells (hBLT1-WT, hBLT1-ΔCyto, hBLT1-Ala, and hBLT1-Asp/Glu) stimulated with various concentrations of LTB4. Each experiment represents an analysis of 3 × 10^6 cells. The cells were detached from monolayer using Versene and incubated with fresh normal medium for 3–4 h. After incubation, cells were washed twice with 1× PBS containing 0.1% BSA. The cells were resuspended in 1× PBS containing 1% BSA at the concentration of 3 × 10^6/ml. The 3 × 10^6 cells were loaded with 1.2 μl of pluronic acid (200 mg/ml in Me2SO) and 1.5 μl of Indo-1 (1 μM solution) and incubated for 30 min at 37 °C. After incubation, the cells were washed twice with Hanks’ balanced salt solution containing PBS + 0.1% BSA. The cells (1 × 10^6 cells/ml) were resuspended in Hanks’ balanced salt solution containing 1 mM CaCl2. The response to various concentrations of ligand was recorded using a spectrophotometer (F2500, Hitachi, San Jose, CA).

Real-time Fluorescence Microscopy—The cells were transfected with expression vectors encoding proteins tagged either with GFP or RFP into RBL-2H3 cells/mouse embryonic fibroblasts (MEF) by electroporation. The cells were grown for 24 h and replated on glass bottom dishes (thickness of glass was 0.17 mm) and allowed to adhere for 1 h at 37 °C. The cells were washed with RPMI 1640 media without phenol red and observed using an inverted x60 objective lens. The fluorescence images were captured using a Nikon Inverted Microscope Eclipse TE300. All of the images were captured by cool snap HQ digital B/W CCD (Roper Scientific) camera. A Lamda 10-2 optical filter changer (Sutter Instrument Company) was used to capture sequential images in different fluorescence wavelengths (for RFP, Texas Red exciter (572 nm) and 4′,6-diamidino-2-phenylindole/FITC/Texas Red emitter (460, 520, and 600 nm); for GFP, FITC exciter (492 nm) and emitter (500–540 nm)). All of the images shown were analyzed on Metamorph 6.0 software. All of the images shown are representative of at least 10 independent experiments collected from at least three separate experiments.

RESULTS

Phosphorylation-dependent Desensitization of hBLT-1—Phosphorylation-defective or phosphomimic mutants of hBLT1 were generated by substituting the 14 Ser/Thr residues in the C-terminal tail with Ala (hBLT1-Ala) or with Asp/Glu (hBLT1-Asp/Glu) (Fig. 1A) and stably expressed to similar levels in RBL-2H3 cells (Fig. 1B). A stable cell line expressing a mutant truncated at amino acid 306 at the C terminus of hBLT1 (hBLT1-ΔCyto) was also generated. Competition ligand binding experiments (Fig. 1C) indicated the affinity to LTβ4 of hBLT1-WT (0.7 nM) is comparable to that seen in neutrophils and in other BLT1-transfected cell lines (3, 19). The similar levels of expression and binding affinities for hBLT1-Ala (1.28 nm) and hBLT1-Asp/Glu (1.31 nm) suggest that these properties of BLT1 remain unaltered by the above mutations in the C-terminal tail. hBLT1-ΔCyto showed a 10-fold decrease in ligand binding affinity relative to the hBLT1-WT receptors (Fig. 1C). Stable cell lines expressing a RFP at the C terminus of hBLT1 or the mutants were also generated and showed similar functional activities as native receptors (see below).

LTβ4 and phorbol 12-myristate 13-acetate stimulated the phosphorylation of hBLT1-WT (Fig. 2, lanes 1–3) or hBLT1-ΔCyto, hBLT1-Ala, or hBLT1-Asp/Glu (Fig. 2, lanes 4–6) by severalfold but not in alanine substitution mutants of this receptor, hBLT1-Ala or hBLT1-Asp/Glu (lanes 7–11). None of these agents stimulated detectable phosphorylation of the hBLT1-Asp/Glu or hBLT1-ΔCyto even after prolonged exposures (data not shown), indicating that phosphorylation of hBLT1 is limited to serine/threonine residues in the cytoplasmic tail. Similar phosphorylation profile of the RFP fusion of hBLT1 suggests normal folding of the hBLT1-RFP and also indicates that RFP itself does not contribute any ligand-dependent or independent phosphorylation sites. The increase in the molecular mass of the protein reflects the additional ~20 kDa contributed by RFP.

Ligand-dependent chemotaxis of hBLT1-WT and mutants was measured as described under “Experimental Procedures” (Fig. 3A). Whereas hBLT1-WT and hBLT1-Ala showed a typi-
cal bell-shaped curve, the phosphomimic mutant, hBLT1-Asp/Glu and hBLT1-Asp/Glu, showed very poor responses at low concentrations of LTB4. However, comparable chemotaxis responses were seen at higher concentrations of LTB4. A similar dose response profile was also observed for the release of β-hexosaminidase with the phosphomimic hBLT1-Asp/Glu responding very weakly at low concentrations of LTB4 but reaching the same maximal response as hBLT1-WT (Fig. 3B). In contrast, the phosphorylation-defective hBLT1-Ala activated a stronger response of β-hexosaminidase release than hBLT1-WT. Consistent with the lack of ligand-induced phosphorylation, the hBLT1-ΔCyto also showed enhanced β-hexosaminidase release compared with hBLT1-WT.

Fig. 4 shows the dose response profiles for calcium mobilization by hBLT1-WT, hBLT1-Ala, and hBLT1-Asp/Glu cells. None of these cells showed any response to vehicle (ethanol) control. LTB4 induced a rapid and transient release of intracellular calcium in hBLT1-WT cells. hBLT1-Ala resulted in a more sustained calcium response at all of the concentrations of LTB4 (Fig. 4B). Similar sustained calcium responses were also seen with hBLT1-ΔCyto but at concentrations consistent with its lower affinity for LTB4 (data not shown). The hBLT1-Asp/Glu failed to release any calcium at lower concentrations of ligand, although the same maximal response was observed at higher concentrations of LTB4 (Fig. 4C). The cumulative data for the calcium release is shown in Fig. 4D where hBLT1-Ala curve shifted to the left side, suggesting the release of higher calcium at lower concentrations of ligand compared with hBLT1-WT. In contrast to hBLT1-Ala, the hBLT1-Asp/Glu mutant curve showed a significant shift to the right side. The results from all of the functional activities suggest that the phosphorylation-deficient hBLT1-Ala is a hyperactive mutant lacking desensitization, whereas the phosphomimic hBLT1-Asp/Glu is behaving as a constitutively desensitized receptor.

Phosphorylation-independent β-Arrestin Translocation and Internalization of BLT-1—To determine the role of phosphorylation in ligand-dependent β-arrestin association and internalization, cell lines stably expressing hBLT1-WT or mutants were transiently transfected with β-arrestin1-GFP. Live imaging of these cells revealed the completely unexpected observation that ligand-induced phosphorylation is not a critical event...
Regulation of Leukotriene B4 Receptors

A hBLT1-ΔCyto-RFP construct was also generated to determine the role of the cytoplasmic tail in β-arrestin interaction and internalization. Although significant surface expression was observed, much of the hBLT1-ΔCyto-RFP remained in the cytoplasm. This could be due to the exact location of RFP at the end of the eighth helix in BLT1, resulting in incomplete processing and membrane translocation. Despite this limited surface expression, the addition of LTBr resulted in clear ligand-induced translocation of β-arrestin to the plasma membrane (Fig. 7).

β-Arrestin Is Required for BLT-1 Internalization—To determine the role of β-arrestin in BLT1 internalization, we expressed hBLT1-RFP in the presence or absence of β-arrestin1-GFP in β-arrestin−/− or in β-arrestin+/− MEFs derived from β-arrestin1/β-arrestin2 double knockout mice and their littermate wild type animals (20). Ligand-induced receptor internalization and β-arrestin translocation were examined in these cells. There was no detectable ligand-dependent internalization of the receptor when hBLT1-RFP was transfected into β-arrestin−/− MEF cells (Fig. 8, left panels). However, receptor internalization was observed when β-arrestin1-GFP was co-transfected along with hBLT1-RFP in β-arrestin−/− cells (Fig. 8, middle panels). The ligand-dependent receptor internalization can be seen in the β-arrestin+/− MEF cells (Fig. 8, right panels). These data suggest that BLT1 internalization is critically dependent on β-arrestin but cell type-independent.

DISCUSSION

Receptor phosphorylation has long been recognized as an important regulatory mechanism for G-protein-coupled receptors (12). In most GPCRs, receptor phosphorylation uncouples G-protein activation from ligand occupancy. β-Arrestin interaction with the phosphorylated receptors facilitates this desensitization process and initiates ligand-induced internalization (22). The results presented here delineate two significant findings regarding the role of phosphorylation in the regulation of the high affinity LTBr receptor, BLT1. First, the use of phosphorylation-defective and phosphomimic mutants showed that phosphorylation regulates G-protein signaling without altering ligand binding properties. Second, the interaction of β-arrestin with ligand-occupied receptors and internalization of hBLT1 are independent of receptor phosphorylation.

Because multiple serine/threonine residues on the cytoplasmic domains of GPCRs could be substrates for G-protein-coupled receptor kinase and mediate desensitization, we have modified all of the serine/threonine residues on the cytoplasmic tail of the hBLT1. Multiple functional activity assays allowed for clear distinctions to be made between the two substitution mutants and the WT receptors. The same levels of stable expression and similar ligand binding profiles indicate that the substitution mutations did not alter either surface expression or binding affinity. This is in contrast to the hBLT1-ΔCyto that showed decreased affinity for LTBr binding in our studies and other cytoplasmic tail truncation mutants that showed increased affinity for LTBr in the experiments reported by Okuno et al. (9). The different truncation end points and the presence of the eighth helix in the cytoplasmic tail of BLT1 and its interaction with the membrane probably account for the altered ligand binding in these distinct truncation mutants.

Because all of the phosphorylation sites in the cytoplasmic tail were altered as might be expected, either the substitution mu-
tants or the C-tail truncation mutant showed any ligand-dependent or basal phosphorylation (Fig. 2). The WT and the mutants of BLT1 as RFP fusion proteins also showed a similar pattern of ligand- and phorbol 12-myristate 13-acetate-induced phosphorylation. Previous studies with the /H9252-adrenergic receptors fused to GFP showed normal signaling, phosphorylation, desensitization, and re-sensitization.

**FIG. 4.** Intracellular calcium release. RBL cells expressing hBLT1-WT (A), hBLT1-Ala (B), and hBLT1-Asp/Glu (C) loaded with Indo-1 were induced with various concentrations (black line, 0.1 nM; red line, 0.3 nM; green line, 1.0 nM; yellow line, 3.0 nM; blue line, 10 nM; and pink line, 100 nM) of LTB₄, and the Ca²⁺ mobilization was measured. D, dose response curves of percent calcium release for hBLT1-WT and its mutants.

**FIG. 5.** LTB₄-induced changes in localization of β-arrestin. RBL cell lines expressing similar levels of the indicated WT or mutant BLT-1 receptors were cotransfected with β-arrestin-GFP. After the addition of LTB₄ (0 time), green fluorescence images were collected every 10 s for 30 min using appropriate filters. Rapid (1 min) translocation of β-arrestin-GFP was seen with BLT1-WT. A delayed but comparable translocation of β-arrestin-GFP was also observed with all of the phosphorylation-defective BLT-1 mutants. With time, vesicular distribution of β-arrestin-GFP was seen with both native and mutant receptors.
and internalization profiles as the native receptors, indicating the presence of this fluorochrome at the C terminus did not have an impact on the basic properties of the GPCRs (23).

A significant observation in the current studies is that, in multiple functional assays, the phosphomimic hBLT1-Asp/Glu mutant showed greatly reduced activities at concentrations of

![Graph showing LTB₄-induced co-localization of hBLT1 and β-arrestin](image)

**Fig. 6.** LTB₄-induced co-localization of hBLT1 and β-arrestin. β-Arrestin-GFP (15 μg) was co-transfected with hBLT1-WT-RFP or with hBLT1-Ala-RFP or with hBLT1-Asp/Glu-RFP into RBL-2H3 cells. After the addition of LTB₄ (0 time), red and green fluorescence images were collected every 10 s for 70 min using appropriate filters as described under “Experimental Procedures.” With time, co-localization of β-arrestin-GFP and receptor RFP (yellow) was seen with both native and mutated receptors. The right panel shows fluorescence quantification with the different hBLT1 constructs as indicated. The average red and green fluorescence intensities at five different membrane (red outside and green outside) and cytoplasmic locations (green inside) were measured by Metamorph software at different time intervals and plotted as percent maximum.

![Graph showing LTB₄-induced changes in β-arrestin localization](image)

**Fig. 7.** LTB₄-induced changes in β-arrestin localization. β-Arrestin-GFP (15 μg) was co-transfected along with hBLT1-ΔCyto-RFP into RBL-2H3 cells. After the addition of LTB₄ (0 time), red and green fluorescence images were collected every 10 s for 30 min using appropriate filters as described under “Experimental Procedures.” The red (receptor), green (β-arrestin), and overlay of the two images at 0, 5, and 15 min are shown. Much of the hBLT1-ΔCyto-RFP remained in the cytoplasm, even in the absence of stimulation. Clear ligand-induced translocation of β-arrestin-GFP is seen at 5 and 15 min (middle panels).
LTB₄ where both the hBLT1-WT and hBLT1-Ala showed maximal responses (Figs. 3 and 4). Chemotaxis, calcium mobilization, and endocytosis all were at only 10–20% of the maximal responses seen at the same ligand concentration with hBLT1-WT or hBLT1-Ala. This apparent lack of activity is unrelated to the receptor occupancy, because the binding affinities for LTB₄ are essentially identical for hBLT1-WT or hBLT1-Ala and hBLT1-Asp/Glu (Fig. 1). Thus, the phosphomimic mutants must have reduced efficacy in activating the cytoplasmic effectors. Previous studies with other chemokine receptors, CXCR2, CXCR4, and n-formyl peptide, and platelet-activating factor receptors have shown that phosphorylation-defective mutants activate enhanced calcium mobilization and exocytosis (24–28). The observation that phosphorylation-defective mutants are hyperactive and the phosphomimic mutant has the opposite effect on signaling indicate that phosphorylation not only has an effect on desensitization of the receptor to a second stimulation but also has a direct impact on the initial response of the receptor to ligand. Despite this decrease in the efficacy, the phosphomimetic receptor was able to activate similar maximal responses as the native receptors. Thus, receptor phosphorylation will limit the response at low concentrations of the ligand, but at sufficiently high ligand concentrations, the maximal initial responses will be reached even with phosphorylated receptors. In this case, the shut down of the functional activity of the receptor might occur at downstream signaling events. Regulator of G protein signaling proteins and phospholipase C offer such targets for desensitization of the chemottractant receptor responses (29, 30).

Ligand-induced phosphorylation of GPCRs is known to result in rapid uncoupling of receptors from G-proteins followed by translocation of β-arrestin to membranes. The β-arrestin complex with the receptors is then internalized through coated pits via dynamin-dependent and -independent mechanisms (22). It was also shown that receptor-β-arrestin complexes act as scaffolds for assembling signaling complexes (31). The results presented here demonstrate that translocation of β-arrestin to membranes and its association with BLT1 receptors are receptor phosphorylation-independent events. The membrane translocation of β-arrestin-GFP and its eventual distribution in endocytic vesicles in cell lines stably expressing the hBLT1 or its phosphorylation mutants suggest that phosphorylation is not a requirement for β-arrestin association with BLT1 (Fig. 5). Changes in ligand-induced distribution of red and green fluorescence at various time points in cells co-expressing RFP receptors and β-arrestin-GFP (Figs. 6 and 7 and supplemental videos) and co-localization of the receptors and β-arrestin both with WT and mutant receptors further support the notion that β-arrestin translocation and internalization of BLT1 are independent of receptor phosphorylation. Distinct types of β-arrestin and GPCR interactions led to the description of class A and class B receptors. Although the class A receptors bind β-arrestin transiently and internalize without it, the class B receptors associate with β-arrestin more tightly and internalize together with it (32, 33). The BLT1 receptors may resemble more class A receptors with the distinction that phosphorylation is not required for β-arrestin association or internalization.

These observations raise questions regarding the probable mechanisms for BLT1 and β-arrestin interaction and the consequences of such interactions in the absence of receptor phosphorylation. Although most GPCRs require phosphorylation for β-arrestin interaction, phosphorylation-independent β-arrestin association was demonstrated in the case of protease-activated receptor-1 and human leutropin receptors (34, 35). In contrast, the interaction of CCR5 receptors with β-arrestin was completely phosphorylation-dependent (36). Thus, both receptor phosphorylation and ligand-induced conformational changes could contribute to β-arrestin interaction with ligand-occupied receptors. In the case of BLT1, it could be argued that the contribution from receptor phosphorylation is minimal if at all present and that ligand-induced conformational change is likely to be the primary determinant for β-arrestin interaction. BLT1-β-arrestin interaction not only occurred in the absence of receptor phosphorylation but also when much of the C-tail was deleted, indicating that the site of β-arrestin association is elsewhere on the cytoplasmic interface of the receptor. This is in contrast with the constitutively recycling chemokine receptor D6 that also interacts with β-arrestin in a phosphorylation-independent manner but needs an acidic region on the cytoplasmic tail (37). In this regard, it is interesting to note in studies by Okuno et al. (9) that a mutation of dleucine motif within the eighth helix to alanine (L304A/L305A) in BLT1 resulted in a hyperactive receptor despite the presence of all of the phosphorylation sites. This mutant showed increased and sustained calcium mobilization. Gaudreau et al. (11) also observed that mutation of the same dleucine motif resulted in complete inhibition of ligand-induced receptor internalization as well as enhanced phosphatidylinositol 1,4,5-trisphosphate accumulation. It is not known whether BLT1 (L304A/L305A) mutant undergoes ligand-induced phosphorylation. Because the hBLT1-ΔCyto examined in the present studies retained this dleucine motif, it is possible that it might serve as a β-arrestin interaction site. Further studies with more proximal mutants of BLT1 as well as β-arrestin mutants using video microscopy methods are likely to unravel molecular determinants of BLT1-β-arrestin interaction. It is interesting to note that the phosphomimic mutant of BLT1 displayed a constitutive desensitization phenotype but is not constitutively associated with β-arrestin. Indeed, a similar delay in the kinetics of β-arrestin interaction with BLT1 occurred with both phosphorylation-deficient as well as phosphomimic mutants. This finding suggests that desensitization of signaling pathways is unrelated to β-arrestin association of hBLT1-Asp/Glu mutant. Partial phosphorylation of n-formyl peptide receptors was shown to reduce G-protein activation in the absence of association with β-arrestin (38). Thus, desensitization may not be the primary function of β-arrestin.
The use of β-arrestin-deficient fibroblasts clearly showed the lack of receptor association upon ligand stimulation with endocytic vesicles in these cells and identified a clear requirement for arrestins to mediate this process. Recent studies (9) suggest that BLT1 is not internalized when surface expression is measured following ligand stimulation. In contrast, Gaudreau et al. (11) observed ~30% internalization of BLT1 receptors. We have also observed very little internalization at room temperature where the Okuno experiments (9) were conducted but consistently have seen ~30% internalization of receptors in different assays including live cell imaging conducted out at 37 °C. Thus, the observed differences in BLT1 internalization between Okuno experiments and ours were probably a function of temperature where the internalization was measured and/or due to the cell type, CHO/HEK in Okuno experiments versus RBL cells in our experiments. It should also be noted that the mutants studied in Okuno experiments disrupted the eighth helix, whereas all of the mutants including hBLT1ΔCyt examined in the present studies retained an intact eighth helix.

A recent study by Chen et al. (39) suggests that agonist-induced internalization of BLT1 does not require arrestins. A lack of co-immunoprecipitation of arrestin and receptors upon ligand stimulation and absence of co-localization of receptors and arrestins in fixed cells with platelet-activating factor receptors providing positive controls were used to conclude that arrestin and BLT1 interaction is not essential for internalization. They have also failed to see the inhibition of BLT1 internalization by dominant negative mutants of β-arrestin. However, excellent discussion of these results highlighted the limitations of the methods and conclusions (21). In particular, it was suggested that weak and transient interactions would not be identified by the methods used in the studies by Chen et al. (21). Indeed, based on their results, it could easily be concluded that interaction of β-arrestin with platelet-activating factor receptors is very strong relative to BLT1-arrestin interactions. The limited internalization (~30%) of BLT1 observed by us and Gaudreau et al. (11) also supports the notion of weak β-arrestin-BLT1 interaction. Whereas positive results (i.e. inhibition of function) with dominant negative mutants are easier to deduce, negative results (lack of an effect) could be due to reasons other than the lack of interactions. In particular, relative levels of endogenous and expressed dominant negative mutants as well as strength of β-arrestin-BLT1 interactions are likely to adversely influence the results. Three separate lines of evidence presented here demonstrated an interaction between arrestin and BLT1 and its requirement for BLT1 internalization. First, arrestin translocates to membranes upon LB4 treatment in stable cell lines expressing BLT1 but not parental RBL cells. Second, live cell real-time imaging of both receptor and arrestin double-labeled cells clearly showed interaction between arrestin and BLT1 (Figs. 6 and 7 and supplemental videos), and finally, in arrestin-deficient MEF cells, BLT1 is not associated with vesicles but co-localizes with externally expressed arrestin. These results define a critical role for receptor phosphorylation in BLT1 signaling but suggest a phosphorylation-independent β-arrestin-mediated internalization mechanism.

Acknowledgment—We thank Dr. Robert J. Lefkowitz for the generous gift of β-arrestin-deficient mouse embryo fibroblasts.

REFERENCES
1. Ford-Hutchinson, A. W., Bray, M. A., Doig, M. V., Shipley, M. E., and Smith, M. J. (1980) Nature 286, 264–265
2. Samuelson, B., Dahlen, S. E., Lindgreen, J. A., Rouzer, C. A., and Serhan, C. N. (1987) Science 237, 1171–1176
3. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., and Shimizu, T. (1997) Nature 387, 620–624
4. Subbarao, K., Jala, V. R., Mathis, S., Suttles, J., Zacharias, W., Ahamed, J., Ali, H., Tseng, M. T., Haribabu, B. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 369–375
5. Yokomizo, T., Kato, K., Terawaki, K., Izumi, T., and Shimizu, T. (2000) J. Exp. Med. 192, 421–431
6. Brink, C., Dahlen, S. E., Drazen, J., Evans, J. F., Hay, D. W., Nicosia, S., Serhan, C. N., Shimizu, T., and Yokomizo, T. (2003) Pharmacol. Rev. 55, 195–227
7. Jala, V. R., and Haribabu, B. (2004) Trends Immunol. 25, 315–322
8. Haribabu, B., Ziehe, D. V., Fidgen, B. C., Richardson, R. M., Ali, H., and Snyderman, R. (1999) J. Biol. Chem. 274, 37087–37092
9. Okuno, T., Ago, H., Terawaki, K., Miyano, M., Shimizu, T., and Yokomizo, T. (2003) J. Biol. Chem. 278, 41500–41509
10. Gaudreau, R., Le Goul, C., Venna, M. H., Stankova, J., and Rola-Pleszcynski, M. (2000) J. Biol. Chem. 277, 31567–31576
11. Gaudreau, R., Beaulieu, M. E., Chen, Z., Le Goul, C., Lavigne, P., Stankova, J., and Rola-Pleszcynski, M. (2004) J. Biol. Chem. 279, 10338–10345
12. Fisher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) Annu. Rev. Biochem. 67, 653–692
13. Luttrell, L. M., and Lefkowitz, R. J. (2002) J. Cell Sci. 115, 455–465
14. Poonson, S. B., Downey, W. E. III, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) Science 271, 363–366
15. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
16. Haribabu, B., Hook, S. S., Selbert, M. A., Goldstein, E. G., Tomhave, B. H., Edelman, A. M., Snyderman, R., and Means, A. R. (1995) EMBO J. 14, 3679–3686
17. Eriksson, L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. (1997) J. Biol. Chem. 272, 27497–27500
18. Ali, H., Richardson, R. M., Tomhave, E. D., DuBose, R. A., Haribabu, B., and Snyderman, R. (1994) J. Biol. Chem. 269, 24551–24563
19. Ali, H., Richardson, R. M., Haribabu, B., and Snyderman, R. (1999) J. Biol. Chem. 274, 6027–6030
20. Kohout, T. A., Lin, F. T., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 1601–1606
21. van Koppen, C. J., and Jakobs, K. H. (2004) Mol. Pharmacol. 66, 365–367
22. Lefkowitz, R. J., and Whalen, E. J. (2004) Annu. Rev. Biochem. 73, 6760–6766
23. Barak, L. S., Ferguson, S. S. G., Zhang, J., Martenson, C., Meyer, T., and Caron, M. G. (1997) Mol. Pharmacol. 51, 177–184
24. Richardson, R. M., Haribabu, B., Ali, H., and Snyderman, R. (1996) J. Biol. Chem. 271, 28717–28724
25. Richardson, R. M., Fidgen, B. C., Haribabu, B., Ali, H., and Snyderman, R. (1998) J. Biol. Chem. 273, 6280–6286
26. Oppermann, M. (2004) J. Biol. Chem. 279, 1201–1210
27. Ali, H., Sozanni, S., Fisher, I., Barr, A. J., Richardson, R. M., Haribabu, B., and Snyderman, R. (1998) J. Biol. Chem. 273, 11012–11016
28. McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000) Science 287, 1754–1757
29. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) J. Biol. Chem. 275, 17201–17210
30. Min, L., Galet, C., and Ascussi, M. (2002) J. Biol. Chem. 277, 7092–7101
31. Chen, C. H., Paine, M. M., and Trejo, J. (2004) J. Biol. Chem. 279, 12020–12031
32. Oppermann, M. (2004) Cell. Signal. 16, 1201–1210
33. Galliera, E., Jala, V. R., Trent, J. O., Bonecchi, R., Signorelli, P., Lefkowitz, R. J., Mantovani, A., Locati, M., and Haribabu, B. (2004) J. Biol. Chem. 279, 25590–25597
34. Bennett, T. A., Foutz, T. D., Gurevich, V. V., Sklar, L. A., and Prosser, E. R. (2001) J. Biol. Chem. 276, 49195–49203
35. Chen, Z., Gaudreau, R., Le Goul, C., Rola-Pleszcynski, M., and Stankova, J. (2004) Mol. Pharmacol. 66, 377–386
