In the present report, we investigated the effect of ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50) expression on the agonist-induced internalization of the thromboxane A_2 receptor (TPα receptor). Interestingly, we found that EBP50 almost completely blocked TPβ receptor internalization, which could not be reversed by overexpression of G protein-coupled receptor (GPCR) kinases and arrestins. Because we recently demonstrated that EBP50 can bind to and inhibit Gαq, we next studied whether Gαq signaling could induce TPβ receptor internalization, addressing the long standing question about the relationship between GPCR signaling and their internalization. Expression of a constitutively active Gαq mutant (Gαq-R183C) resulted in a robust internalization of the TPβ receptor, which was unaffected by expression of dominant negative mutants of arrestin-2 and -3, but inhibited by expression of EBP50 or dynamin-K44A, a dominant negative mutant of dynamin. Phospholipase Cβ and protein kinase C did not appear to significantly contribute to internalization of the TPβ receptor, suggesting that Gαq induces receptor internalization through a phospholipase Cβ- and protein kinase C-independent pathway. Surprisingly, there appears to be specificity in Gα protein-mediated GPCR internalization. Gαq-R183C also induced the internalization of CXCR4 (Gαq-coupled), whereas it failed to do so for the β1-adrenergic receptor (Gαq-coupled). Moreover, Gαq-R201C, a constitutively active form of Gαq, had no effect on internalization of the TPβ, CXCR4, and β2-adrenergic receptors. Thus, we showed that Gα protein signaling can lead to internalization of GPCRs, with specificity in both the Gα proteins and GPCRs that are involved. Furthermore, a new function has been described for EBP50 in its capacity to inhibit receptor endocytosis.

G protein-coupled receptor (GPCR) signaling cascades are regulated by different molecular mechanisms. Many proteins have been shown to regulate the different GPCR-related signaling pathways through the direct regulation of the G protein activity (1). Following agonist binding, many GPCRs undergo agonist-induced phosphorylation, internalization, and down-regulation, resulting in a decrease of their responsiveness (desensitization) (2). Our studies are mainly interested in the mechanisms that regulate the signaling and the internalization of the thromboxane A_2 receptor (TP receptor). Two TP receptor isoforms were identified which are generated by the alternative splicing of a single gene, TPα (343 amino acids) and TPβ (407 amino acids), which share the first 328 amino acids (3, 4). Previous experiments performed by Parent et al. (5) demonstrated that only TPβ, but not TPα, undergoes agonist-induced and tonic (constitutive) internalizations, which are dictated by distinct motifs in the C terminus of the TPβ receptor. Different experiments showed that agonist-induced production of the second messenger inositol phosphate by the TP receptors results from the activation of the Gαq11 subunits (6). Gαq-mediated production of inositol phosphates involves the stimulation of phospholipase Cβ (PLC-β) isoforms (7). More recently, we have shown that the ezrin-radixin-moesin-binding phosphoprotein EBP50 regulates the Gαq signaling pathway of the TP receptors (8). We showed that EBP50 binds preferentially to the active form of Gαq and thus diminishes the Gαq-induced inositol phosphates production on the one hand by inhibiting the interaction of the activated Gαq with PLCβ-1, and on the other hand by preventing the coupling of the TP receptors to Gαq (8).

EBP50 (also known as NHERF1), a 55-kDa phosphoprotein, was first identified as a cofactor essential for protein kinase A-mediated inhibition of Na^+/H^+ exchanger isoform 3 (9). EBP50 contains two PDZ domains (PDZ1 and PDZ2) implicated in multiple protein-protein interactions, and an ERM domain, which binds to the actin-associated ERM proteins (ezrin, radixin, moesin, and merlin) (10, 11). EBP50 has been found to interact with a variety of proteins, and these interactions are involved in a growing range of functions including the assembly of signaling complexes, receptor recycling, and transport of membrane proteins to the cell surface (12). In fact, Cao et al. (13) have shown that EBP50 participates in GPCR trafficking by demonstrating its involvement in the recycling back to the cell surface of the β2-adrenergic receptor following its agonist-induced internalization. However, there is still no evidence that EBP50 can regulate the internalization of GPCRs. It has long been assumed that ligand binding to GPCRs is the ultimate control to their internalization and trafficking.
However, recent studies bring about a new concept of the tight regulation of GPCRs internalization pathways by diverse signaling cascades (14). In fact, many signaling molecules including the small G proteins Rho, Rab, and ARF6, as well as different phosphoinositide molecules, have been shown to regulate the endocytic pathways by either controlling the actin cytoskeleton organization or by mediating clathrin-coated pit formation, as reviewed by Cavalli et al. (14). One of the questions often asked in GPCR regulation is whether GPCR signaling is necessary for GPCR internalization. Evidence gathered over the years by several groups suggests that there is no clear answer to that question and that it may depend on the GPCR and the signaling pathways that are studied. Several GPCR mutants unable to activate their cognate G proteins could still internalize, whereas the opposite was also observed, signaling-deficient GPCRs were unable to undergo agonist-induced internalization (15). In most cases, however, effects of the mutations on the receptor signaling were determined for only one signaling pathway even though a given GPCR can sometimes couple to more than one type of Go subunit. Moreover, different studies using constitutively active GPCR mutants suggest a possible link between receptor signaling and internalization. Indeed, many such mutations seem to affect cellular distributions of various GPCRs by decreasing their cell surface expression levels, resulting in their intracellular localization (16, 17), which can be reversed by the addition of an inverse agonist (17). Even if there is evidence of a link between the constitutive signaling activity of some GPCRs and their constitutive internalization, it is not yet clear whether this internalization is the result of activation of the signaling cascades or simply of the active conformation adopted by the constitutively active receptors. In other words, it is not well understood whether GPCR conformational changes triggered by the binding of the agonist are sufficient and necessary to the internalization of different GPCRs or whether the subsequent activation of the signaling cascades plays an important role in initiating the internalization pathways. On the other hand, it has been shown that heterologous activation of protein kinase C (PKC) results in the phosphorylation and internalization of the CXCR4 (18) and the β-opioid receptors (19). These studies suggested that activation of intracellular signaling induces the agonist-independent internalization of some GPCRs. However, there is still no or little evidence for the direct involvement of Go protein signaling in the control of internalization pathways.

In the present study, we demonstrate that Go protein signaling can induce GPCR internalization. In fact, inhibition of Goq signaling almost completely blocked the agonist-induced internalization of the Tβ3 receptor. Surprisingly, we show that there is specificity in this phenomenon. Indeed, Goa signaling leads to Goq−, but not Goq−, coupled receptor internalization, whereas Goa signaling does not trigger internalization of the GPCRs that we studied. The Goa-mediated internalization was determined to occur by an arrestin-, PLC-, and PKC-independent, but dynamin-dependent mechanism and was completely abrogated by EB50.

**EXPERIMENTAL PROCEDURES**

**Materials**—Goa111, Goa, and PLCβ1-specific polyclonal antibodies were from Santa Cruz Biotechnology. Hemagglutinin (HA)-specific monoclonal antibody was from Babco. ECL reagents were purchased from Amersham Biosciences. Flag-specific monoclonal antibody was purchased from Sigma, whereas the fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and Texas Red goat anti-rabbit antibodies were purchased from Molecular Probes. The goat anti-mouse alkaline phosphatase-conjugated antibody and the alkaline phosphatase substrate kit were purchased from Bio-Rad.

**Cell Culture and Transfection**—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO2.

Transient transfections of HEK293 cells grown to 75–90% confluence were performed using FuGene 6™ according to the instructions from the manufacturer. Empty pCDNA3 vector was added to keep the total DNA amount added per plate constant. Cells grown on 60-mm plates were transfected using 6 μg of total DNA vectors.

**Receptor Cell Surface Expression and Internalization Assays**—Cell surface expression and internalization of CXCR4, Tβ3, and β2AR were assessed by ELISA experiments using transiently transfected HEK293 cells as we have described previously (5). Briefly, 1.2 × 106 cells were grown overnight in 60-mm plates. The cells were then transfected with empty pcDNA3 vector or cotransfected with pcDNA3-HA-Tβ3, pcDNA3-Flag-Tβ3, pcDNA3-Flag-Tβ3/Y339A, pcDNA3-HA-CXCR4, pcDNA3-HA-β2AR, or pcDNA3-Flag-β2AR with pcDNA3-HA-EB50, pcDNA3-Flag-β2AR (Y339A) (a receptor mutant specifically deficient in constitutive internalization) and either an empty pcDNA3 vector or with pcDNA3-Myc-CXCR4, pcDNA3-HA-EB50(Y339A), pcDNA3-dynamin-K44A, pcDNA3-HA-EBP50(PDZ1-PDZ2), pcDNA3-Gαq, pcDNA3-ARRESTIN-3, pcDNA3-HA-R183C, pcDNA3-HA-EBP50(Y339A), pcDNA3-dynamin-K44A, pcDNA3-ARRESTIN-3, pcDNA3-ARRESTIN-2(319–418) (DN). Transfected cells were maintained as described above for 24 h. Thereafter, 2 × 105 cells were transferred to 24-well plates precoated with 0.1 mg/ml poly-l-lysine and were maintained for an additional 24 h. To assess the agonist-induced or PMA-induced internalization, the transfected cells were washed once with phosphate-buffered saline followed by a 2-h incubation at 37 °C with 100 nM U46619 or 1 μM PMA in Dulbecco’s modified Eagle’s medium, except for the cells that were cotransfected with the receptors described above and pcDNA3-Gαq, R183C. The cells were then fixed with 3% formaldehyde plus 1% BSA at room temperature. The cells were then washed three times with TBS and nonspecific binding blocked with TBS containing 1% BSA for 45 min at room temperature. The cells were then incubated with either a HA-specific (Babco) or the Flag M1-specific (Sigma) monoclonal antibody at a dilution of 1:1000 in TBS/BSA for 1 h at room temperature. Three washes with TBS buffer followed, and cells were briefly roblotted for 15 min at room temperature. Incubation with goat anti-mouse conjugated alkaline phosphatase diluted 1:1000 was carried out for 1 h at room temperature. The cells were incubated with an alkaline phosphatase-conjugated goat anti-mouse antibody for 1 h at room temperature. The cells were washed three times with TBS, and a colorimetric alkaline phosphatase substrate was added as specified by the manufacturer. Colorimetric readings were carried out using a Titertek Multiskan MCC/340 spectrophotometer. Cells transfected with pcDNA3 were studied concurrently to determine background. All experiments were done in triplicate.

**Immunofluorescence Microscopy—**Tβ3/Y339A (a receptor mutant defective in constitutive internalization) internalization was assessed with immunofluorescence microscopy. 1.2 × 106 HEK293 cells were grown overnight in 60-mm plates as described above. The cells were then transfected with an empty pcDNA3 vector or cotransfected with pcDNA3-HA-Tβ3/Y339A (a receptor mutant specifically deficient in constitutive internalization) and either an empty pcDNA3 vector or pcDNA3-Gαq, R183C and maintained overnight as described above. The transfected cells were fixed with 3% formaldehyde plus 1% Triton X-100 plus PBS for 15 min at room temperature. Non-specific binding was blocked with 0.1% Triton X-100 plus PBS containing 5% nonfat dry milk for 30 min at room temperature. Goat anti-mouse FITC-conjugated secondary antibody (Molecular Probes) was then added at a dilution of 1:200 for 1 h at room temperature. The cells were washed six times with permeabilization buffer, washed with PBS followed by a wash left at room temperature for 30 min. The cells were then incubated for 1 h at 4 °C with the primary antibody and further grown overnight. Cells were incubated with an HA-specific monoclonal antibody (1:500 dilution) for 1 h at 4 °C in Dulbecco’s modified Eagle’s medium supplemented with 1% BSA, followed by a 2-h incubation at 37 °C. The cells were then fixed with 3% paraformaldehyde plus PBS for 30 min at room temperature, washed with PBS/0.1% Triton X-100 plus PBS for 10 min at room temperature. Non-specific binding was blocked with 0.1% Triton X-100 plus PBS containing 5% nonfat dry milk for 30 min at room temperature. Goat anti-mouse FITC-conjugated secondary antibody (Molecular Probes) was then added at a dilution of 1:200 for 1 h at room temperature. The cells were washed six times with permeabilization buffer, washed with PBS followed by a wash left at room temperature for 30 min. The cells were fixed with 3% paraformaldehyde as described. Coverslips were mounted using Vectashield mounting medium (Vector Laboratories) and examined by immunofluorescence microscopy on a Zeiss Axioskop2 microscope using a 40× objective. Images were collected using QED Camera software and processed with Adobe Photoshop.

**Phosphorylation—**Receptor phosphorylation of the Tβ3 receptor was assessed essentially as we described previously (18). 3.5 × 105 HEK293 cells were grown overnight in six-well plates as described above and transfected the following day with an empty pcDNA3 vector or with pcDNA3-Myc-Tβ3. Forty-eight hours after transfection, the cells were washed once with TBS and incubated for 2 h with growth-factor-free Dulbecco’s modified Eagle’s medium supplemented with 1 μCi/ml of 32P3 at 37 °C (PerkinElmer Life Sciences). The cells were then incubated in the presence or absence of 1 μM GF109203X (a PKC inhibitor) for 30 min at 37 °C, followed by a 30-min stimulation with 1 μM PMA at 37 °C. The cells were then washed twice with cold TBS and harvested in 800
Regulation of GPCR Internalization by \( \text{Go}_{q} \) Signaling

\( \mu l \) of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM Na<sub>2</sub>PO<sub>4</sub>, 5 mM EDTA) supplemented with protease inhibitors (9 nM pepstatin, 9 nM antipain, 10 nM leupeptin, and 10 nM chymostatin) (Sigma). After the cells were incubated in lysis buffer for 60 min at 4 °C, the lysates were clarified by centrifugation for 20 min at 14,000 rpm at 4 °C. Four \( \mu l \) of Myc-specific monoclonal antibody were added to the supernatant. After a 60-min incubation with rotation at 4 °C, 50 \( \mu l \) of 50% protein A-agarose pre-equilibrated in lysis buffer was added, followed by an overnight incubation at 4 °C with rotation. Samples were then centrifuged for 1 min in a microcentrifuge and washed three times with 1 ml of ice-cold lysis buffer. Immunoprecipitated proteins were eluted by addition of 50 \( \mu l \) of SDS sample buffer followed by a 30-min incubation at room temperature. The immunoprecipitates were resolved on 10% SDS-PAGE gels. The gels were dried and subjected to autoradiography at 80 °C.

**Inositol Phosphate Measurements in Cells**—Inositol phosphate measurements were performed as described previously (24). HEK293 cells (2 \( \times \) 10<sup>5</sup>) were grown overnight in 12-well plates. The cells were then cotransfected as described above with the indicated constructs. The cells were labeled the following day for 18–24 h with 4 \( \mu \)Ci/ml myo-[<sup>3</sup>H]inositol in Dulbecco’s modified Eagle’s medium (high glucose, without insulin). The cells were washed once in PBS and incubated in prewarmed Dulbecco’s modified Eagle’s medium (high glucose, without inositol) supplemented with 0.5% BSA, 20 mM Heps, pH 7.5, and 20 mM LiCl for 10 min. Cells were then stimulated for 30 min with 100 nM U46619 in the case of the TP receptor-transfected cells. After stimulation, the medium was removed and the reactions were terminated by addition of 0.8 ml of 0.4 mM chilled perchloric acid. Cells were then collected in Eppendorf tubes and 0.5 volume of 0.72 N KOH, 0.6 M KOHCO<sub>3</sub> added. Tubes were mixed and centrifuged for 5 min at 14,000 rpm in a microcentrifuge. Inositol phosphates were separated on Bio-Rad AGI-8 columns. Total labeled inositol phosphates were then measured by liquid scintillation counting.

**Data Analysis**—Data are presented as means ± S.E. of at least three independent experiments. Statistically significant differences among groups were assessed by t test for paired samples, with \( p < 0.05 \) sufficient to reject the null hypothesis.

**RESULTS AND DISCUSSION**

**EBP50 Inhibits TPβ Receptor Internalization**—We have already shown that EBP50 inhibits TPβ signaling through the \( \text{Go}_{q} \) pathway by both preventing the receptor coupling to \( \text{Go}_{q} \) and by sequestration of GTP-bound \( \text{Go}_{q} \), blocking it from binding and activating PLCβ-1 (8). We then investigated the role of EBP50 in the agonist-induced internalization of an HA-tagged TPβ receptor by the use of ELISA experiments, as we described previously (5). Surprisingly, we have found that EBP50 almost completely inhibited the agonist-induced internalization of the TPβ receptor, as shown in Fig. 1A. In fact, EBP50 inhibited the internalization of the TPβ receptor more efficiently than the dominant negative mutant form of dynamin, dynamin-K44A, which is deficient in GTP binding and known to block internalization of several membrane proteins (20). We had already demonstrated that agonist-induced internalization of the TPβ receptor is dynamin-dependent (5), and dynamin-K44A was thus included as a control to compare with.

EBP50 has been shown to be involved in the control of the fate of internalized membrane proteins such as the \( \beta_{3} \)-adrenergic receptors (13) by promoting their recycling to the cell surface membrane. We were thus interested in determining whether the apparent lack of TPβ receptor internalization in presence of EBP50 was not in fact caused by an increase in receptor recycling to the cell surface. Cao et al. (13) have shown that the ERM domain of EBP50 was necessary for promoting the recycling of the \( \beta_{3} \)-adrenergic receptor and that a dominant negative mutant of EBP50 lacking the ERM domain was unable to enhance the recycling of this receptor. Similarly, data later published showed that the ERM domain of EBP50 was also necessary for the recycling of the \( \kappa \)-opioid receptor (21). Thus, we used a PDZ1-PDZ2 construct (lacking the ERM domain) of EBP50 to investigate its effect on TPβ receptor internalization. As seen in Fig. 1A, we have found that this EBP50 mutant is still able to inhibit TPβ receptor internalization, strongly suggesting that the inhibition of TPβ receptor internalization by EBP50 could not be explained by an increase in the recycling of the TPβ receptor. We then verified the effect of EBP50 and its PDZ1-PDZ2 construct (lacking the ERM domain) on the agonist-induced internalization of the \( \beta_{3} \)-AR receptor to confirm that EBP50 was acting appropriately in our hands. The results obtained showed that, in the presence of EBP50, the agonist-induced internalization of the \( \beta_{3} \)-AR receptor decreased by 50%, whereas cotransfection of the PDZ1-PDZ2 construct (lacking the ERM domain) had no effect on \( \beta_{3} \)-AR receptor internalization (data not shown), indicating that the effect of EBP50 on the agonist-induced internalization of \( \beta_{3} \)-AR results from an increase in its recycling at the cell surface, as was elegantly shown by Cao et al. (13). Moreover, as we illustrate in Fig. 1B, pretreatment of cells with monensin had no effect on neither the EBP50 inhibition nor, as expected, on the dynamin-K44A inhibition of the agonist-induced TPβ receptor internalization. Monensin is known to inhibit cell surface membrane recycling activity (22), and we have shown it to inhibit TPβ receptor recycling in our laboratory. Data presented so far strongly suggest that EBP50 inhibits the TPβ receptor internalization, rather than promoting its recycling back to the cell surface. To further demonstrate that EBP50 acts at the level of TPβ receptor internalization, we performed immunofluorescence microscopy experiments. In Fig. 1C, cells transfected with pcDNA3-HATPβ alone or with pcDNA3-HATPβ and pcDNA3-EBP50myc were labeled at 4 °C with an anti-HA antibody prior to internalization experiments to allow for detection of only the receptors that are trafficking from the cell surface, as we described previously (5). After agonist stimulation, it can be seen that, in absence of EBP50, the TPβ receptors are internalized in intracellular compartments (Fig. 1C, top left panel). In contrast, receptor-associated fluorescence remained at the cell surface in presence of EBP50 following agonist treatment (Fig. 1C, bottom left panel), and this, for all the time points observed between 0 and 2 h of receptor stimulation (data not shown). Fig. 1C further illustrates that EBP50 blocks TPβ receptor internalization. These observations were surprising in light of the known role of EBP50 in receptor recycling and transport of membrane proteins to the cell surface. Indeed, it has never been shown that EBP50 can inhibit the internalization of a GPCR. Therefore, we were then interested in investigating the mechanism involved in the inhibition of the TPβ receptor internalization by EBP50.

**GRKs and Arrestins Cannot Overcome the Inhibition of Internalization by EBP50**—Because we showed that the EBP50 inhibition mechanism of the TPβ receptor internalization did not involve the control of the TPβ receptor recycling, we then investigated the effect of EBP50 on the machinery known to participate in GPCR internalization. We previously demonstrated that the agonist-induced internalization of the TPβ receptor is GRK- as well as arrestin-dependent (5). Therefore, we performed an ELISA experiment using HEK293 cells stably transfected with HA-tagged TPβ receptors transfected with pcDNA3, pcDNA3-EBP50, pcDNA3-GRK2, pcDNA3-GRK5, pcDNA3-arrestin-3, and both pcDNA3-EBP50 and either pcDNA3-GRK2, pcDNA3-GRK5 or pcDNA3-arrestin-3. Fig. 2 shows that 40% of TPβ receptors undergo agonist-induced internalization when the cells are transfected with an empty pcDNA3 vector. Overexpression of GRK2, GRK5, and arrestin-3 in the absence of EBP50 increased TPβ receptor internalization to 50, 54, and 60%, respectively. Similar results were obtained previously for GRK2 and arrestin-3 (5). It is the first observation regarding...
Regulation of the TPβ receptor by GRK5, and thus identifies the TPβ receptor as a putative substrate for GRK5. However, Fig. 2 also shows that EBP50 inhibition of TPβ receptor internalization could not be reversed by overexpression of GRK2, GRK5, or arrestin-3. Overexpression of arrestin-2 also failed to reverse the inhibition brought by EBP50 (data not shown). This result suggests that EBP50 probably acts through a mechanism other than the GRK- and arrestin-mediated internalization pathway.

**Fig. 1. Inhibition of TPβ receptor internalization by EBP50.** A, HEK293 cells were transiently transfected with pcDNA3-HATPβ and pcDNA3, pcDNA3-EBP50, pcDNA3-PDZ1-PDZ2, pcDNA3-PDZ1, pcDNA3-PDZ2, or pcDNA3-dynamin-K44A constructs. The percentage of cell surface receptor loss following a 2-h incubation with 100 nM U46619 was measured by ELISA as described under “Experimental Procedures.” B, HEK293 cells transiently co-expressing the HA-tagged TPβ receptor and either EBP50 or dynamin-K44A were preincubated with monensin (50 μM) for 30 min followed by a 2-h incubation with 100 nM U46619. The percentage of cell surface receptor loss was measured as above. C, immunofluorescence analysis of TPβ receptor internalization inhibition by EBP50 expression in HEK293 cells. The cells were transiently cotransfected with pcDNA3-HATPβ and either pcDNA3 or pcDNA3-EBP50myc. Cells were incubated with a HA-specific monoclonal antibody at 4 °C for 1 h, followed by a 2-h incubation at 37 °C in the presence of 100 nM U46619. The cells were then fixed and incubated with a Myc-specific monoclonal antibody. Receptors and EBP50myc were visualized by incubating the cells with FITC-conjugated anti-mouse and Texas Red goat anti-rabbit secondary antibodies, respectively. The cells were then processed for immunofluorescence detection as described under “Experimental Procedures.” Inhibition of receptor internalization by EBP50 was observed for all the time points observed between 0 and 2 h of agonist stimulation.
Gaq Signaling Induces TPβ Receptor Internalization—As mentioned above, we have recently shown that EBP50 interferes with the TPβ receptor signaling through the Gaq pathway (8). EBP50 mediates the interaction of several membrane proteins with the cell cytoskeleton via its ERM domain (11). However, the ERM domain is not involved in the regulation of Gaq signaling. Indeed, the PDZ1-PDZ2 peptide and the individual PDZ1 and PDZ2 domains of EBP50 are sufficient for Gaq binding and the inhibition of Gaq-mediated signaling (8). Interestingly, the PDZ1-PDZ2 construct and the individual PDZ1 and PDZ2 domains of EBP50 are not only capable of, but are sufficient for, the inhibition of TPβ receptor internalization (Fig. 1A). These observations prompted us to investigate the possible role of Gaq signaling in the regulation of the TPβ receptor internalization. Surprisingly, we have found that TPβ receptor-expressing HEK293 cells transfected with a constitutively active Gaq mutant (Gaq-R183C) exhibited a very low level of TPβ receptor expression at the cell surface (Fig. 3). In fact, more than 80% of TPβ cell surface expression was lost when Gaq-R183C was expressed compared with TPβ receptor-expressing cells transfected with pcDNA3. Moreover, loss of TPβ receptor expression at the cell surface was proportional to the amount of transfected pcDNA3-Gaq-R183C DNA (data not shown). Interestingly, the loss of TPβ receptor expression induced by Gaq-R183C was prevented by the co-expression of dynamin-K44A (Fig. 3). Dynamin-K44A inhibits the agonist-induced internalization of the TPβ receptor (Fig. 1A), as well as the internalization of several GPCRs (20). Thus, it appears that the reduced surface expression of the TPβ receptors in presence of Gaq-R183C is the result of an agonist-independent internalization process. Because EBP50 binds Gaq-R183C, preventing it from activating Gaq downstream signaling (8), we investigated the effect of EBP50 on the Gaq-R183C-induced internalization of TPβ receptors. We observed a full recovery of cell surface expression of TPβ receptors in HEK293 cells cotransfected with pcDNA3-HA-TPβ, pcDNA3-Gaq-R183C, and pcDNA3-EBP50 in comparison to HEK293 cells cotransfected with pcDNA3-HA-TPβ, pcDNA3-Gaq-R183C, and pcDNA3 (Fig. 3). This result indicates that EBP50 inhibits the Gaq-R183C-induced internalization of the TPβ receptor.

To further demonstrate (and visualize) that Gaq-R183C induces internalization of the TPβ receptor, we performed immunofluorescence microscopy experiments using HEK293 cells cotransfected with pcDNA3-HA-TPβ-Y339A and either pcDNA3-Gaq-R183C or an empty pcDNA3 vector. The wild-type TPβ receptor exhibits constitutive internalization, and its use would complicate the visual interpretation of the results in this particular case. Instead, we are taking advantage of the TPβ-Y339A mutant, which is specifically deficient in constitutive internalization (23). Receptors were labeled with an anti-HA antibody at 4 °C for 1 h prior to internalization experiments to follow the receptors trafficking from the cell surface, as described for Fig. 1C. The cells were then transferred to 37 °C to allow internalization to take place, and the receptors visualized by incubating with a goat anti-mouse FITC-conjugated antibody. Fig. 4 shows that only the cells that were cotransfected with both pcDNA3-HATPβ-Y339A and pcDNA3Gaq-R183C exhibited intracellular fluorescence, whereas the receptor-associated fluorescence remained at the cell surface in cells cotransfected with pcDNA3-HATPβ-Y339A and pcDNA3. This result visually confirmed that Gaq-R183C induces an agonist-independent internalization of cell surface TPβ receptors, as was determined in Fig. 3 with the ELISA data.

Although the inhibition of Gaq-R183C-induced internalization of the TPβ receptor by dynamin-K44A most probably occurs at the level of the pinching off of forming endocytic intracellular vesicles from the cell surface (20), the inhibition of TPβ receptor internalization by EBP50 is likely the result of its capacity to interfere with Gaq signaling. Taken together, these results suggest that Gaq-mediated signaling is involved in TPβ internalization, because on one hand Gaq-R183C is able to induce TPβ receptor internalization and on the other hand TPβ receptor agonist activation results mainly in the activation of the Gaq pathway (6). Furthermore, EBP50, which efficiently inhibits the activation of the Gaq signaling pathway, is also able to inhibit both the agonist-promoted and Gaq-R183C-induced internalization of TPβ receptors, indicating that activation of Gaq is involved in triggering the internalization process.

Protein Kinase C Activation Induces TPβ Receptor Internalization—Gaq activation induces inositol phosphate production by PLCβ isozforms leading to activation of PKC, which mediates the phosphorylation of a broad range of cellular substrates (24). We next studied the possible role of Gaq-mediated activation of PLCβ and PKC in initiating TPβ receptor internalization. Interestingly, we have found that TPβ receptors undergo agonist-independent internalization in HEK293 cells cotransfected with pcDNA3-HATPβ and pcDNA3 following PMA treatment (Fig. 5A). As expected, PMA-induced internalization of TPβ
receptors was inhibited by the expression of dynamin-K44A (Fig. 5A). However, expression of EBP50 did not prevent the PMA-induced TPβ receptor internalization (Fig. 5A). In fact, this result was expected because we showed EBP50 to bind and inhibit Goq, which is upstream of PKC activation, and should thus have no effect on direct activation of PKC by PMA (8). The incapacity of EBP50 to block PMA-induced internalization of TPβ receptors supports our idea that it does not interfere with the internalization machinery per se. Therefore, our data strongly suggest that EBP50 inhibition of agonist-induced internalization of TPβ receptor is more likely a result of its direct inhibition of Goq signaling. These observations also indicate that receptor-, as well as Goq-R183C-, induced activation of PKC could be involved in internalization of the TPβ receptor. PKA activation and heterologous activation of PKC are already known to promote the phosphorylation and internalization of both δ-opioid and CXCR4 receptors (18, 19). To verify whether PKC activation is involved in initiating agonist-promoted TPβ receptor internalization, we looked at the effect of the PKC-specific inhibitor GF109203X in this process. Fig. 5B shows that TPβ receptor agonist-induced internalization was partially inhibited following pretreatment of cells with 1 μM GF109203X. The efficiency of PKC inhibition by GF109203X was verified by carrying out a receptor phosphorylation assay. HEK293 cells transfected with pcDNA3 or pcDNA3-mycTPβ were labeled with 32P, and subjected to immunoprecipitation experiments with an anti-Myc-specific monoclonal antibody following a 1 μM PMA treatment for 30 min at 37 °C, in presence or absence of GF109203X (Fig. 5C). As can be seen, PMA treatment of the receptor-transfected cells caused a significant phosphorylation of TPβ receptor, whereas pretreatment of cells with GF109203X completely prevented the PKC-mediated phosphorylation of the receptor, showing that PKC was efficiently inhibited in our system. Thus, it seems that activation of PKC is not a major player in the control of the agonist-induced internalization of the TPβ receptor. Moreover, Goq-R183C-induced internalization was not prevented with PKC inhibitors (data not shown).

The possible role of PLCβ activation in the regulation of the agonist-promoted internalization of the TPβ receptor was then evaluated. We have found that pretreatment of cells with U73122, a PLCβ-specific inhibitor, could not prevent the agonist-induced internalization of the TPβ receptor (Fig. 5B), whereas it reduced TPβ receptor-mediated inositol phosphate production by 80% (Fig. 5D). Thus, it appears that PLCβ activation is also not a major contributor in triggering the TPβ receptor internalization process. Taken together, our results suggest that agonist-dependent internalization of TPβ receptor is strongly dependent on Goq signaling because direct inhibition of Goq by EBP50 virtually abrogated completely both agonist-promoted and Goq-R183C-induced internalization, but that there are mechanisms other than PLCβ- and PKC-associated pathways involved. In this regard, it is interesting to note that Goq and Goq-coupled receptors were recently shown to activate RhoA and its downstream signaling events independently of second messengers production (25–27). Furthermore, Rho signaling pathways are known to be involved in the regulation of endocytic trafficking, as reviewed by Cavalli et al. (14). This research avenue is currently pursued in our laboratory.

Goq, but Not Goq Signaling Regulates the Internalization of the TPβ Receptor—The results presented above brought the first evidence for a direct regulation of Goq-coupled receptor internalization by Goq signaling. As a means to assess the specificity or generality of Goq protein signaling in triggering GPCR internalization, we performed ELISA experiments to investigate the effect of a constitutively active Goq-R201C mutant on the cell surface expression of TPβ receptors. Fig. 6A reveals that co-expression of TPβ receptor with Goq-R201C did not affect the cell surface expression of TPβ receptors in comparison to the expression level observed in HEK293 cells expressing TPβ receptor alone. Functionality of the Goq-R201C protein was confirmed in our system by cAMP measurements (data not shown). Only co-expression of Goq-R183C with the TPβ receptor was able to induce a decrease in its cell surface expression. This result shows that, contrary to Goq signaling, Goq-mediated pathways do not regulate the internalization of the TPβ receptor. Thus, a specificity in Goq-mediated internalization of the TPβ receptor seems to exist, which is very interesting because this receptor is also known to couple to Goq (Ref. 6 and data not shown).

GPCR Specificity of the Goq-R183C-induced Internalization—The effect of Goq signaling on the regulation of the internalization of CXCR4 receptor, another Goq-coupled receptor, was then studied. We performed ELISA experiments using HEK293 cells cotransfected with pcDNA3-HA-CXCR4 and either pcDNA3-Goq-R183C or an empty pcDNA3 vector. Inter-

![Image of a bar graph showing the effect of Goq-R183C and Goq on TPβ receptor internalization.](image-url)

**Fig. 3. Goq-R183C expression induces TPβ receptor internalization.** HEK293 cells were transiently cotransfected with pcDNA3-HATPβ and pcDNA3, pcDNA3-Goq-R183C alone, or pcDNA3-Goq-R183C with either pcDNA3-dynamin-K44A or pcDNA3-EBP50. Cell surface expression of the TPβ receptor was measured by ELISA as described under "Experimental Procedures."
Interestingly, we observed a significant decrease (85%) in the cell surface expression of HA-CXCR4 in HEK293 cells expressing \( \text{G}_\text{o}_q \text{-R183C} \) compared with the cells cotransfected with pcDNA3 (Fig. 6B). These results show that a direct activation of \( \text{G}_\text{o}_q \) signaling cascade by expression of \( \text{G}_\text{o}_q \text{-R183C} \) in HEK293 cells promotes an agonist-independent internalization of both TP\( \beta \) and CXCR4 receptors, both \( \text{G}_\text{o}_q \)-coupled receptors.

This is intriguing as it suggests that heterologous activation of \( \text{G}_\text{o}_q \) signaling could result in the internalization of perhaps several \( \text{G}_\text{o}_q \)-coupled receptors (see below). However, because we only used two \( \text{G}_\text{o}_q \)-coupled receptors in our studies, more experiments will need to be done to examine how our observations will apply to other \( \text{G}_\text{o}_q \)-coupled receptors.

To further investigate the specificity of \( \text{G}_\text{o}_q \text{-R183C} \)-induced internalization, we used a different approach. We transfected HEK293 cells with pcDNA3-HATP\( \beta \text{Y339A} \) and pcDNA3-\( \text{G}_\text{o}_q \text{-R183C} \) (A), pcDNA3-HATP\( \beta \text{Y339A} \) and pcDNA3 (B), and pcDNA3 alone (C). Cells were incubated with a HA-specific monoclonal antibody at 4 °C for 1 h, followed by a 2-h incubation at 37 °C, and then processed for immunofluorescence detection as described under “Experimental Procedures.” Top and bottom panels in A and B show different cells from the same experiment. Results are representative of three independent experiments.

**Fig. 4. Immunofluorescence analysis of TP\( \beta \text{Y339A} \) internalization induced by \( \text{G}_\text{o}_q \text{-R183C} \) expression in HEK293 cells.** HEK293 cells were transfected with pcDNA3-HATP\( \beta \text{Y339A} \) and pcDNA3-\( \text{G}_\text{o}_q \text{-R183C} \) (A), pcDNA3-HATP\( \beta \text{Y339A} \) and pcDNA3 (B), and pcDNA3 alone (C). Cells were incubated with a HA-specific monoclonal antibody at 4 °C for 1 h, followed by a 2-h incubation at 37 °C, and then processed for immunofluorescence detection as described under “Experimental Procedures.” Top and bottom panels in A and B show different cells from the same experiment. Results are representative of three independent experiments.
internalization, we performed ELISA experiments using HEK293 cells cotransfected with pcDNA3-HA-β2AR and pcDNA3-Gαq-R183C, pcDNA3-Gαq-R201C, or an empty pcDNA3 vector. Fig. 6C shows that Gαq-R183C co-expression did not affect the cell surface expression of the β2AR in comparison to cells cotransfected with pcDNA3-HA-β2AR and pcDNA3. Because β2AR is a Gαq-coupled receptor, we looked at the effect of the constitutively active mutant Gαq-R201C on the cell surface expression of β2AR. The results shown in Fig. 6C demonstrate that Gαq-R201C expression did not induce internalization of the β2AR, but that it actually slightly increased β2AR cell surface expression, for which we have no explanation yet. These data suggest that neither Gαq-R183C nor Gαq-R201C induce internalization of the β2AR. Therefore, Gαq signaling seems to promote the internalization of specific GPCRs, possibly those which are coupled to Gαq. The use of other GPCRs and active Gα proteins to further characterize the specificity of Gα-mediated internalization of GPCRs will constitute future interesting studies.

Gαq-R183C-induced Internalization Is Arrestin-independent—We demonstrated that Gαq-R183C constitutive signaling induces internalization of both Gαq-coupled TPβ and CXCR4 receptors. Because agonist-dependent internalization of TPβ is arrestin-dependent (5), we investigated the effect of arrestin-3-(201–409) and arrestin-2-(319–418) (28, 29) dominant negative (DN) mutants on Gαq-R183C-induced internalization of the TPβ receptor. ELISA experiments were performed using HEK293 cells cotransfected with pcDNA3-HATPβ and an empty pcDNA3 vector, or with both pcDNA3-HATPβ and pcDNA3-arrestin-2(DN), pcDNA3-arrestin-3(DN), or an empty pcDNA3 vector. Fig. 7 shows that the Gαq-R183C-induced internalization of the TPβ receptor could not be prevented by co-expression of both arrestin-2(DN) and arrestin-3(DN) mutants. Together, results from Figs. 2 and 7 suggest that internalization of the TPβ receptor by Gαq signaling is an arrestin-independent mechanism. GPCR endocytosis involves diverse molecular mechanisms that can be arrestin-dependent or not (30). Our results also suggest that both arrestin-dependent and arrestin-independent endocytosis pathways could be activated upon agonist stimulation of the TPβ receptor. This is interesting because we reported in a
previous study that the agonist-induced internalization of the TPβ receptor was only inhibited by roughly 50% by expression of arrestin dominant negative mutants (5). We speculated at the time that there could be an alternative route for TPβ receptor endocytosis than the arrestin-mediated pathway. In the present study, we provide part of the answer to that question by showing that Gaq signaling is involved in the internalization of the TPβ receptor in an arrestin-independent manner.

Heterologous Internalization of Gaq-coupled Receptors—

Taken together, our results provide evidence of a new mechanism consisting in Gaq-mediated internalization of some Gaq-coupled receptors. It also raises the question whether heterologous activation of the Gaq signaling pathway by any Gaq-coupled receptor could activate the agonist-independent internalization of another Gaq-coupled receptor present at the same cell surface. In an attempt to answer this question, we performed an ELISA experiment using HEK293 cells cotransfected with pcDNA3-HA-CXCR4 and either pcDNA3-Flag-TPβ or pcDNA3-EBP50, or pcDNA3. The percentage of cell surface CXCR4 receptor loss following a 2-h incubation with 100 nM TP receptor-specific agonist U46619 was measured by ELISA as indicated under “Experimental Procedures.”
Regulation of GPCR Internalization by Go-q Signaling

Fig. 9. Schematic illustration of the internalization mechanisms of the TPβ receptor. According to our previous results (5) and to data presented here, the TPβ receptor appears to undergo agonist-induced internalization through various mechanisms. Upon agonist stimulation, internalization of the receptor is promoted by GRKs and arrestins through a dynamin- and clathrin-mediated pathway. However, dominant-negative mutants of GRKs and arrestins were unable to completely inhibit TPβ receptor internalization (5). Our new data show that there is a GRK- and arrestin-independent component in the internalization of the TPβ receptor, which is mediated by Go-q signaling. PLCβ and PKC activation do not appear to constitute major contributors in Go-q-mediated internalization of the TPβ receptor, as indicated by the thick arrow linking the receptor, Go_q activation, and an unknown pathway leading to receptor internalization in a dynamin-dependent fashion. EBP50, which we showed to bind to Go_q and to inhibit its signaling, efficiently blocked Go_q-induced receptor internalization. Go_q activation promoted Go_q-coupled receptor internalization, whereas Go_q signaling failed to induce receptor internalization (results not shown).

served in HEK293 cells expressing both TPβ and CXCR4 receptors, but not in the cells expressing CXCR4 alone. This experiment demonstrates that activation of the TPβ receptor results in an agonist-independent internalization of the CXCR4 receptor. This result was interesting, but also expected, as we have shown that the internalization of both the TPβ and CXCR4 receptors could be induced by Go_q-R183C. Interestingly, consistent with our hypothesis, heterologous internalization of CXCR4 induced by stimulation of the TPβ receptor was inhibited by coexpression of EBP50 (Fig. 8). Thus activation of the Go_q signaling pathway by agonist stimulation of the TPβ receptor can induce the internalization of the CXCR4 receptor, another Go_q-coupled receptor.

In the present study, we provide new information to the question which has been asked for a long time: is Go protein signaling involved in internalization of GPCRs? Our results determined that Go protein signaling can trigger GPCR trafficking. Indeed, we showed that Go_q signaling can induce internalization of GPCRs. There is specificity in the Go protein that can promote GPCR internalization, as illustrated by the fact that Go_q signaling could not induce internalization of the receptors included in this report. We demonstrated that the Go_q-mediated internalization shows at least some specificity for Go_q-coupled receptors, because β2AR (a Go_q-coupled receptor) internalization was not induced by Go_q signaling. We presented evidence that the Go_q-induced internalization mechanism was PLCβ-, PKC-, GRK-, and arrestin-independent, therefore constituting a GPCR internalization mechanism that has never been appreciated before. One possible link connecting Go_q signaling to GPCR internalization could be the Go_q-mediated activation of Rho-associated pathways (25–27), which are known to participate in the actin cytoskeleton rearrangement and to regulate endocytic processes (14). Interestingly, the TP receptor was recently shown to activate Rho (31). It is unclear as to why in our system, where Go_q signaling was independent of ligand occupancy of the receptor, only specific GPCRs were induced to internalize. This would suggest that receptor-specific sequences are implicated in the recognition by the machinery involved in the Go_q-mediated internalization, which is the subject of current work in our laboratory.

Another interesting finding presented here is that EBP50 strongly inhibited the TPβ receptor internalization. Inhibition of receptor endocytosis is a function that has never been described before for EBP50. Overexpression of GRKs and arrestins could not overcome the EBP50 inhibition of TPβ receptor internalization, indicating that EBP50 does not act at this level. EBP50 most likely inhibits TPβ receptor internalization by binding to Go_q. This is supported by a few observations. First, we know that EBP50 binds Go_q and inhibits TPβ receptor Go_q signaling pathways (8). Second, EBP50 strongly prevented the Go_q-R183C-mediated but not the PMA-induced internalization of the TPβ receptor. Finally, each of the individual PDZ domains (which bind Go_q) of EBP50 inhibited receptor internalization, suggesting that the formation of a multiprotein complex by EBP50 is not necessary, but that sequestration of Go_q is sufficient for inhibition of TPβ receptor endocytosis. A schematic representation of our working model is illustrated in Fig. 9.

Our work was performed in a system where proteins were overexpressed. We have established the specificity of the inter-
action between Goq and the PDZ domains of EBP50 previously (8). The fact that EBP50 cannot block PMA-induced internalization of the receptor supports the idea that EBP50 does not act nonspecifically in the process of internalization. Furthermore, other PDZ domain-containing proteins that were tested, including NHERF2 (an isoform of EBP50) and GIPC, do not bind Goq (8) and do not affect TPβ receptor internalization (data not shown). The physiological consequences and significance of our observations that Goq signaling can lead to “heterologous” or “cross-regulated” internalization of GPCRs is intriguing. It could constitute another step in GPCRs heterologous desensitization, whereas a cell could protect itself from overstimulation of particular signaling pathways. Although we have no doubt that the heterologous Goq-mediated internalization occurs in more physiological conditions, it is hard to imagine that all Goq-coupled receptors expressed at a cell surface will undergo internalization once Goq is activated by a given GPCR. Mechanisms must exist to regulate this phenomenon, such as cellular compartmentalization, receptor-specific sequences, associated proteins conferring further specificity, or a combination of some or all of these possibilities. Clearly, much work remains to be done to fully appreciate the mechanisms and consequences of Goq protein-mediated internalization of GPCRs.

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