Regulation of GTP-binding Protein $\alpha_q$ ($G\alpha_q$) Signaling by the Ezrin-Radixin-Moesin-binding Phosphoprotein-50 (EBP50)∗

Although ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50) is a PDZ domain-containing protein known to bind to various channels, receptors, cytoskeletal elements, and cytoplasmic proteins, there is still very little evidence for a role of EBP50 in the regulation of receptor signal transduction. In this report, we show that EBP50 inhibits the phospholipase C (PLC)-β-mediated inositol phosphate production of a Gαq-coupled receptor as well as PLC-β activation by the constitutively active Gαq-R183C mutant. Coimmunoprecipitations experiments revealed that EBP50 interacts with Gαq and to a greater extent with Gαq-R183C. Agonist stimulation of the thromboxane $A_2$ receptor (TP receptor) resulted in an increased interaction between EBP50 and Gαq, suggesting that EBP50 preferentially interacts with activated Gαq. We also demonstrate that EBP50 inhibits Gαq signaling by preventing the interaction between Gαq and the TP receptor and between activated Gαq and PLC-β1. Investigation of the EBP50 regions involved in Gαq binding indicated that its two PDZ domains are responsible for this interaction. This study constitutes the first demonstration of an interaction between a G protein α subunit and another protein through a PDZ domain, with broad implications in the regulation of diverse physiological systems.

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to be characterized. Knowing that the TP receptor signaling cascade involves the activation of Goq and the stimulation of the downstream effector PLC-β, we investigated the effect of EBP50 on the signaling of the agonist-stimulated TP receptor through the Goq pathway. Surprisingly, our experiments revealed that EBP50 regulates the Goq signaling pathway by preferentially binding through its PDZ domains to the activated Goq, and by preventing its interaction with PLC-β. Furthermore, we show that EBP50 also interferes in the TP receptor-Gαq coupling. This novel interaction between PDZ domains and Goq had broad implications in intracellular signaling regulation. Growing evidence demonstrates the role of EBP50 in a wide variety of physiological events. The regulation of these events could possibly be modulated by the Goq-EBP50 interaction.

EXPERIMENTAL PROCEDURES

Reagents—The Myc, Goq32, and PLCβ1-specific polyclonal antibodies were from Santa Cruz Biotechnology. Myc-specific monoclonal antibody was a gift from Dr. J. Stankova (Université de Sherbrooke). Hemagglutinin (HA)-specific monoclonal antibody was from Babco, and EBP50-specific monoclonal antibody was from BD Biosciences. ECL reagents were purchased from Amer sham Biosciences. Protein A-agarose and FuGENE 6™ were purchased from Roche Molecular Biochemicals.

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. Cells were transfected with pcDNA3-gene or pcDNA3-Gαq-EBP50, pcDNA3-NSC-Gαq, pcDNA3-Gαq-R183C, pcDNA3-Myc-Tpα1, pcDNA3-Myc-Tpα2, pcDNA3-PDZ2-HA, pcDNA3-PDZ2-EBP50, pcDNA3-PDZ2-ERD-HA, and pcDNA3-ERM-HA, in the different combinations indicated under the “Results.” Transfected cells were maintained as described above for 48 h. The cells transfected with Tpα or Tpβ were incubated for 0–30 min at 37 °C in the presence or the absence of 100 μM U46619 prior to harvesting. The cells were then rinsed with ice-cold phosphate-buffered saline and harvested in 800 μl of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM Na3PO4, 5 mM EDTA) supplemented with protease inhibitors (9 mM pepstatin, 9 mM antipain, 10 mM leupeptin, and 10 mM chymostatin) (Sigma). After the cells were incubated in lysis buffer for 80 min at 4 °C, they were clarified by centrifugation for 20 min at 14,000 rpm at 4 °C. One to four μg of specific monoclonal or polyclonal antibodies were added to the supernatant. After 60 min of incubation at 4 °C, 50 μl of 50% protein A-agarose pre-equilibrated in lysis buffer was added, followed by an overnight incubation at 4 °C. Samples were then centrifuged for 1 min in a microcentrifuge and washed three times with 1 ml of lysis buffer. Immunoprecipitated proteins were eluted by addition of 50 μl of SDS sample buffer followed by a 30-min incubation at room temperature. Initial lysates and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotsting using specific antibodies.

Inositol Phosphates Measurement in Cells—Inositol phosphates measurements were performed as described previously (24). 2 × 105 HEK293 cells 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 h and then cotransfected as described above with the indicated constructs. HEK293 cells were grown overnight in 12-well plates. The cells were labeled inositol phosphates were then measured by liquid scintillation counting.

RESULTS

EBP50 Inhibits TP Receptor-mediated PLC-β Activation—To investigate the effect of EBP50 on TPαβ(δ) signaling, we measured total inositol phosphate production in HEK293 cells cotransfected with either pcDNA3-HA-Tpα or pcDNA3-HA-Tpβ with an empty pcDNA3 vector (100% control) or pcDNA3-EBP50 following a U46619 (a stable TP receptor agonist) stimulation. GRK2 has been shown to inhibit the inositol phosphate response of both TP receptor isoforms (26) and was thus included in these experiments as a positive control. Fig. 1A shows that EBP50 expression results in a significant loss in the agonist-induced inositol phosphate production by both Tpα and Tpβ, reaching only 41 and 45% of their maximal response, respectively. Receptor expression was determined to be 1.2 ± 0.2 pmol of receptor/mg of protein for each construct combination shown in Fig. 1A.

Inhibition of Goq-mediated Signaling by EBP50—Because the TP receptors activate PLC-β through the Goq protein, we looked at whether EBP50 could directly inhibit Goq signaling. Goq-R183C, a GTPase-deficient Goq mutant that leads to the constitutive activation of PLC-β (27), was cotransfected with either an empty pcDNA3 vector (100% control), EBP50, GRK2, GRK5, or GRK6 in HEK293 cells. Although it was shown that GRK2 inhibited Goq-R183C-mediated activation of PLC-β (58), it was thus used as an experimental control. Surprisingly, EBP50 inhibits significantly the inositol phosphate production induced by Goq-R183C-mediated activation of PLC-β. Indeed, we observed a loss of more than 60% of the inositol phosphate production when Goq-R183C was cotransfected with EBP50 as compared with its transfection with an empty vector (Fig. 1B). These results suggest that EBP50

Construction of Epitope-tagged EBP50 Domains—The pcDNA3-EBP50-HA construct was a kind gift of Dr. Mark Von Zastrow (University of California, San Francisco). The different HA-tagged domains of EBP50 (PDZ1, PDZ2, ERM, PDZ1-PDZ2, and PDZ2-ERM) were obtained by the use of polymerase chain reaction using the Expand High Fidelity PCR System (Roche Molecular Biochemicals), subcloned in the pcDNA3 vector, and the integrity of the coding sequence was confirmed by dideoxy sequencing.

Recombinant EBP50 Production—cDNA encoding for EBP50 was subcloned in the pRSET A vector (Invitrogen), and the construct was used to produce a His6-tagged recombinant EBP50 in the Escherichia coli K-12 PR745 by following the manufacturer’s instructions. The recombinant EBP50 was purified using the QIAexpressionist™ kit (Qiagen, Canada) as indicated by the manufacturer. The purified recombinant EBP50 was analyzed by SDS-PAGE and was immunoblotted by using an EBP50-specific monoclonal antibody as described above. The purified recombinant EBP50 was later used in the Goq interaction and GTPase assays.

Binding Assay—The His-EBP50-Goq-R183C binding assay was performed using recombinant EBP50 produced as described above. His-EBP50 (50 μg) was incubated for 30 min at room temperature with 100 μl of Ni-NTA resin (Qiagen, Canada) in binding buffer (20 mM Tris-HCl, pH 8, 2 mM MgSO4, 6 mM Mg-mercaptoethanol, 100 mM NaCl, 0.05% Nonidet P-40, 5% glycerol). Thereafter, the resin was washed three times with the binding buffer. The Ni-NTA-bound EBP50 was then incubated for 1 h at 4 °C with cell extracts from HEK293 cells transfected with either pcDNA3-Goq-R183C or with pcDNA3 in binding buffer. The binding reactions were then washed three times with binding buffer. SDS sample buffer was added to the binding reactions, and the tubes were boiled for 5 min. The binding reactions were analyzed by SDS-PAGE and immunoblotting using the indicated specific antibodies.

GTPase Assay—Goq GTPase activity was assessed in solution using a single turnover assay essentially as described previously (25). After incubation of Goq-R183C in the presence of γ-32PGTP, the GTP-loaded Goq-R183C was filtered in G-25 Sephadex. Thereafter, GTP(γ-32PGTP) was incubated in the absence or the presence of BSA (100 μg), EBP50 (100 ng), EBP50 (250 μg), or control buffer. Reactions were quenched with 20 μl of 5% (w/v) Norit A Charcoal in 50 mM NaH2PO4. The charcoal was pelleted, and the 32P-containing supernatant was counted.
mediates the inhibition of the production of inositol phosphates following TPα and TPβ stimulation by preventing the TP(α/β)-activated Goq from activating PLC-β.

EBP50 Preferentially Interacts with Activated Goq—EBP50 is a PDZ-domain containing protein known for its ability to bind to a wide variety of other proteins. We were thus interested in determining if EBP50 could interact with Goq. Consequently, coimmunoprecipitation experiments were performed in HEK293 cells cotransfected with pcDNA3-EBP50-HA and either Goq, Goq-R183C, or empty pcDNA3. Cell lysates were then incubated with a Goq-specific polyclonal antibody. Blotting of the immunoprecipitation reactions with an HA-specific antibody revealed that EBP50-HA could be coimmunoprecipitated with Goq and Goq-R183C. However, the amount of EBP50-HA that coimmunoprecipitated with Goq-R183C was greater than with Goq (Fig. 2A, upper panel). The presence of a faint EBP50-HA band in absence of overexpressed Goq indicates that endogenous Goq proteins are sufficient for coimmunoprecipitation of EBP50. Immunoblotting results of the cell lysates with the HA-specific antibody showed that EBP50-HA expression levels were similar in the cells transfected with pcDNA3-EBP50-HA (data not shown). The Goq-EBP50 interaction was further verified in cell lysates immunoprecipitated with an EBP50-specific antibody, and the resulting material was blotted with the Goq-specific antibody to visualize the coprecipitated Goq (Fig. 2B, upper panel). As it can be seen, Goq-R183C and Goq could be immunoprecipitated with either endogenous or transfected EBP50. As expected from Fig. 2A, Goq-R183C coimmunoprecipitated with EBP50 in a larger amount than the wild type Goq. The equivalent expression of EBP50 in the cell lysates was confirmed by immunoblotting using an EBP50-specific monoclonal antibody (data not shown). The EBP50-Goq-R183C interaction was further confirmed by running HEK293 cell extracts expressing Goq, Goq-R183C over His-tagged EBP50-purified protein bound to a Ni²⁺ column (Fig. 3). The upper panel of Fig. 3 shows that Goq-R183C binds to EBP50, whereas it does not bind to the free Ni-NTA resin. Furthermore, no binding was observed when cellular extracts from HEK293 transfected with pcDNA3 were used. The lower panel of Fig. 3 shows immunoblotting of the binding reactions with anti-EBP50 monoclonal antibody.

TPβ Promotes the Goq-EBP50 Interaction—The results de-
scribed in Fig. 2 showed that EBP50 coimmunoprecipitated preferentially with Gaα, R183C (a constitutively active form of Gaα) than it did with Gaα (the assumed “inactive” form). Thus, our data strongly suggest that the interaction of EBP50 with the activated form of Gaα could be implicated in the mechanism leading to the inhibition of the agonist-induced inositol phosphate production by TPα. To verify this, immunoprecipitation experiments were performed using HEK293 cells transfected with pcDNA3-Myc-TPα and pcDNA3-EBP50-HA. The cells were incubated with the TP receptor agonist U46619 (500 nM) for different incubation times from 0 to 30 min. Cell lysates were then incubated with the Gaα-specific antibody, and the immunoprecipitation reactions were assessed by immunoblotting with the HA-specific monoclonal antibody. As shown in Fig. 4, only a small amount of EBP50 could be coimmunoprecipitated with Gaα in the absence of TPα agonist stimulation, consistent with results seen in Fig. 2A. However, a greater amount of EBP50 could be coimmunoprecipitated with Gaα after a 5-min TPα stimulation, suggesting that the agonist treatment resulted in the activation of endogenous Gaα leading to an increased interaction with EBP50 (Fig. 4, upper panel). Basal levels of EBP50 coimmunoprecipitated with Gaα when TPβ was stimulated for longer periods, suggesting an inactivation of GTP-bound Gaα and a loss of Gaα-EBP50 interaction. Our data indicate that activation of Gaα by a GPCR results in a transiently increased Gaα-EBP50 interaction. Similarly, activation of Gaα by the TP and β2-adrenergic receptors showed that this Ga protein subunit could also bind transiently to EBP50 when activated, whereas other Ga proteins tested so far (Gaα11, Gaα12/13, and Gaα16) failed to interact with EBP50 in our system, showing a specificity in the EBP50 interaction with Ga subunits.

EBP50 Prevents the TPα/β Receptor-Gaα Interaction—Because EBP50 coimmunoprecipitates with Gaα, we investigated the effect of EBP50 on the TPα(β)-Gaα interaction. HEK293...
cells were cotransfected with different combinations of pcDNA3-Myc-TPβ, pcDNA3-Myc-TPα, pcDNA3-Gαq, pcDNA3-EBP50-HA, and pcDNA3 as described in Fig. 5. The cells were then stimulated or not with 500 nM U46619 for 5 min, and the cell lysates were incubated with a Myc-specific monoclonal antibody. Blotting of the immunoprecipitation reactions with a Gαq-specific polyclonal antibody revealed that, in the absence of EBP50 overexpression, Gαq coimmunoprecipitated with TPα and TPβ demonstrating physical coupling of the receptors to Gαq (Fig. 5, upper panel). After agonist stimulation of TPβ, the amount of coimmunoprecipitated Gαq decreased due to the activation and the subsequent uncoupling of Gαq. This uncoupling was observed in a lesser extent for the TPα receptor. These observations argue for differential TPα/β coupling to Gαq. This difference is not surprising because TPα and TPβ are also subject to differential regulation of other events such as phosphorylation (17) or constitutive and agonist-induced internalization (24, 28). However, in the HEK293 cells transfected with either TPβ or TPα with Gαq and EBP50, EBP50 seemed to inhibit the coupling of both receptors to Gαq, because we observed a significant decrease in the amount of Gαq that could be coimmunoprecipitated with both receptors.

Once more, the negative effect of EBP50 on TPβ coupling to Gαq was more significant than on the TPα coupling to Gαq. Thus, it appears that EBP50 interferes in the receptor-Gαq interaction.

**EBP50 Prevents Activated Gαq from Binding to PLC-β1**—It is well known that the GTP-bound (active) form of Gαq enhances the phospholipase activity of PLC-β1 by directly binding to its C2 carboxyl-terminal domain (29). In order to investigate whether the EBP50-(GTP-Gαq) interaction results in a sequestration of the activated Gαq, thus preventing it from activating the downstream effectors such as PLC-β1, we performed immunoprecipitation experiments of endogenous PLC-β1 in HEK293 cells cotransfected with pcDNA3-Gαq-R183C or pcDNA3-Gαq, and either pcDNA3-EBP50-HA or an empty pcDNA3. The cell lysates were then incubated with a PLC-β1-specific polyclonal antibody. Blotting of the immunoprecipitation reactions with a Gαq-specific antibody revealed that a large amount of Gαq-R183C could be coimmunoprecipitated with PLC-β1 in the absence of transfected EBP50 (Fig. 6A). As we expected, these data indicate that Gαq-R183C interacts with PLC-β1 resulting in the inositol phosphate production shown in Fig. 1B. The interaction between Gαq-R183C and PLC-β1 was almost completely inhibited when EBP50-HA was present. This result demonstrates that EBP50 interferes with the Gαq-dependent activation of PLC-β1 by preventing the GTP-bound Gαq-PLC-β1 interaction. It can also be observed that only Gαq-R183C, and not the inactive form of Gαq, could bind to PLC-β1 (Fig. 6A). Fig. 6D demonstrates that EBP50 also binds to PLC-β1, as was recently shown by Tang et al. (9).

**EBP50 Binds to Gαq-R183C through Its PDZ Domains**—We were next interested in studying the EBP50 domains involved in the interaction with activated Gαq. Different deletion constructs of EBP50 were thus generated, as schematically represented in Fig. 7A. HEK293 cells were transfected with pcDNA3-Gαq-R183C and either of the indicated EBP50 mutant constructs. Cell lysates were then incubated with the Gαq-specific antibody, and the immunoprecipitation reactions were analyzed by blotting with an HA-specific monoclonal antibody.
Fig. 6. EBP50 inhibits the interaction of Goq-R183C with PLC-β1. HEK293 cells expressing Goq-R183C alone or coexpressing Goq-R183C and EBP50-HA were harvested and lysed as described under “Experimental Procedures.” The cell lysates were incubated with a PLC-β1-specific polyclonal antibody followed by incubation with protein A-agarose. The immunoprecipitation (IP) reactions were analyzed by Western blotting with a Goq-specific polyclonal antibody. The results showed that EBP50 prevented the binding of Goq-R183C to PLC-β1 (A). B and C represent the immunoblotting (IB) of the cell extracts with Goq- and PLC-β1-specific polyclonal antibody showing equal amounts of Goq-R183C and PLC-β1, respectively, present in the cell extracts. D, coimmunoprecipitation of EBP50-HA with PLC-β1 as detected by Western blotting of the same immunoprecipitation reactions with the HA-specific monoclonal antibody.

Fig. 7. Identification of the EBP50 domains interacting with Goq-R183C. A, schematic representation of the different EBP50 constructs that were generated for the identification of the domains responsible for the Goq-R183C interaction. B, HEK293 cells coexpressing different HA-tagged EBP50 domains and Goq-R183C were harvested and lysed as described under “Experimental Procedures.” The cell lysates were incubated with a Goq-specific polyclonal antibody followed by incubation with protein A-agarose. The immunoprecipitation (IP) reactions were analyzed by immunoblotting (IB) using an HA-specific monoclonal antibody (upper panel). Expression of each of the EBP50s was confirmed in the cell lysates by immunoblotting with the HA-specific monoclonal antibody (middle panel). Equivalent immunoprecipitation of Goq-R183C in the reactions was verified by Western blotting with a Goq-specific polyclonal antibody (lower panel).
The results obtained have shown that the ERM domain does not coimmunoprecipitate with Goq-R183C (Fig. 7B). On the other hand, PDZ1 and PDZ2 coimmunoprecipitated with Goq-R183C, indicating that these domains are implicated in the EBP50-Goq-R183C interaction. However, the PDZ1-PDZ2 construct seems to coimmunoprecipitate in a greater amount than did each individual PDZ domain (PDZ1 and PDZ2) or the PDZ2-ERM mutant and to a similar level as the full-length EBP50 (Fig. 7B, upper panel). Thus, it appears that the PDZ1-PDZ2 domains are responsible for the full interaction with activated Goq.

PDZ1 and PDZ2 Interfere with the Physical Coupling of the TP Receptor to Goq and with the Activation of PLC-β1 by Goq-R183C—EBP50 is thought to be an important link for several membrane proteins to the actin cytoskeleton through its ERM domain, which is involved in the binding to the cytoskeleton-binding ERM proteins. The results described above have shown that the PDZ1 and PDZ2 domains are involved in the EBP50-(Goq-R183C) interaction, whereas the ERM domain is not. However, we wondered if the ERM domain of EBP50 is important for its inhibition of the Goq binding to PLC-β1 and the TP receptor. HEK293 cells were transfected with pcDNA3-Goq-R183C and either pcDNA3-PDZ1-HA, pcDNA3-PDZ2-HA, or pcDNA3-ERM-HA. The cell lysates were then incubated with a PLC-β1-specific polyclonal antibody. The immunoprecipitation reactions were analyzed by blotting with the Goq-specific antibody. Surprisingly, the PDZ1 and PDZ2 domains inhibited the binding of Goq-R183C to PLC-β1, whereas the ERM domain did not (Fig. 8A).

The effect of each EBP50 domain on the interaction between TPβ and Goq was then investigated. HEK293 cells were transfected with pcDNA3-Myc-TPβ, pcDNA3-Goq-R183C, and either pcDNA3-PDZ1-HA, pcDNA3-PDZ2-HA, or pcDNA3-ERM-HA. The cells were then harvested and lysed as described under "Experimental Procedures." The cell lysates were then incubated with a PLC-β1-specific polyclonal antibody followed by incubation with protein A-agarose. A, Western blotting of the immunoprecipitation (IP) reactions with a Goq-specific polyclonal antibody. B and C, expression of each of the HA-tagged EBP50 domains was confirmed by Western blotting of the cell extracts with an HA-specific monoclonal antibody. IP, immunoprecipitation; IB, immunoblot.

**Fig. 8.** Inhibition of the interaction between Goq-R183C and PLC-β1 by the individual EBP50 domains. HEK293 cells were cotransfected with Goq-R183C and either pcDNA3, PDZ1-HA, PDZ2-HA, or ERM-HA. The cells were then harvested and lysed as described under "Experimental Procedures." The cell lysates were then incubated with a PLC-β1-specific monoclonal antibody. A, Western blotting of the immunoprecipitation samples with a Goq-specific polyclonal antibody. B and C, equal amounts of PLC-β1 and Goq-R183C could be detected in the cell extracts using the PLC-β1-specific and the Goq-specific polyclonal antibodies, respectively. D, the expression of EBP50-HA and the different HA-tagged EBP50 domains was confirmed by Western blotting of the cell extracts with an HA-specific monoclonal antibody. IP, immunoprecipitation; IB, immunoblot.

**Fig. 9.** TPβ receptor-Goq interaction blockade by the PDZ1 and PDZ2 domains of EBP50. HEK293 cells expressing Myc-TPβ and Goq, and either EBP50-HA, PDZ1-HA, PDZ2-HA, or ERM-HA domains of EBP50 were harvested and lysed as described under "Experimental Procedures." The cell lysates were then incubated with a Myc-specific monoclonal antibody followed by incubation with protein A-agarose. A, Western blotting of the immunoprecipitation (IP) reactions with a Goq-specific polyclonal antibody showed that EBP50 and its first and second PDZ domains, but not the ERM domain, interfere with Goq binding to the TPβ receptor. B, equivalent expression of Goq in cell extracts was verified for each reaction by blotting with the Goq-specific polyclonal antibody. C, expression of each of the HA-tagged EBP50 constructs in the cell lysates was confirmed by immunoblotting (IB) with the HA-specific monoclonal antibody. D, quantities of immunoprecipitated Myc-TPβ receptors were the same for all reactions as revealed by Western blotting with the Myc-specific monoclonal antibody.

**EBP50 Has No GAP Activity for Goq***—Finally, we wanted to assess if EBP50 showed any GAP activity for Goq. GDP dissociation, and therefore GTP loading, in the absence of activated GPCR is particularly inefficient compared with kcat for GTP hydrolysis for Goq precluding use of the single turnover assay (30). However, the kcat for GTP hydrolysis of Goq-R183C is significantly reduced allowing GTP loading to occur more efficiently (31). Furthermore, it was recently shown that the GTPase activity of Goq-R183C can be promoted by RGS4 in a single turnover assay (31). Thus, we utilized this assay to monitor the GTPase activity of Goq-R183C in the absence or presence of purified RGS4 or EBP50. Although 100 nM RGS4 promoted rapid GTP hydrolysis releasing up to 7–8 fmol of P1,
GTPase activity (Goq-R183C)

![Figure 10. Regulation of Goq GTPase activity by EBP50. The Goq GTPase activity was determined in solution by incubating Goq R183C(2-32P)GTP in the presence of 100 nM RGS4 (○), 100 nM EBP50 (●), or control buffer (□) for 0–15 min as described under "Experimental Procedures." Reactions were quenched and pelleted, and the 32P-containing supernatant was counted.](image)

100 and 250 nM EBP50 failed to enhance GTP hydrolysis over 15 min of incubation (Fig. 10). Thus, it appears that EBP50 has no ability to function as a GAP for Goq.

**DISCUSSION**

This study demonstrates a direct regulation of the Goq signaling pathway by EBP50. We have shown that EBP50 inhibited the inositol phosphate production induced by TPα and TPβ agonist stimulation. We showed that EBP50 decreased PLC-β-dependent production of inositol phosphates induced by Goq R183C, a constitutively active mutant of Goq. We demonstrated that EBP50 interacts in a greater extent with Goq R183C than with Goq. Moreover, stimulation of the TPβ receptor promoted the Goq-EBP50 interaction, strongly indicating that EBP50 interacts better with the activated Goq. It was observed that, beyond 5 min of agonist stimulation of TPβ, the amount of EBP50 that coimmunoprecipitated with Goq decreased to levels similar to what was observed in the absence of agonist. It is known that the activation of Goq by GPCRs is a rapid process involving the release of GDP by Goq and its exchange for a GTP. Thereafter, rapid hydrolysis of the GTP by the intrinsic GTPase activity of Goq results in the inactivation of the Goq subunit and its return to the inactive GDP-bound state. In addition, rapid desensitization of GPCRs occurs after agonist stimulation resulting in their inability to activate G protein subunits. Taken together, this could explain the loss of EBP50-Goq interaction after prolonged stimulation of TPβ. These results strongly suggest that the regulation of the TP receptors signaling by EBP50 is caused by the binding of the activated Goq to EBP50. However, because activation of the Goq subunit results in its dissociation from the βγ subunits, the strong binding of the active form of Goq to EBP50 could be due to its dissociation from the βγ subunits and not to its GTP-bound active state per se. Arguing against the last statement is the fact that the overexpressed inactive Goq did not interact as well as Goq-R183C with EBP50, unless the endogenous βγ subunits can account for the difference. Furthermore, the activated Goq-EBP50 interaction after receptor stimulation is transient, suggesting that the Goq comes off the EBP50 protein upon GTP hydrolysis, which could indicate that EBP50 indeed binds preferentially to the active form of Goq. More experiments need to be performed to verify if the βγ subunits compete with EBP50 for the binding of Goq.

Our results showed that EBP50 interferes with the coupling of the TP receptors to Goq, which could be explained by the binding of EBP50 to both the active and the inactive forms of Goq, leading to the possible sequestration of Goq. It can be argued that EBP50 could also bind directly to the receptor, as reported for other GPCRs (5), and further interfere in the Goq coupling this way. The EBP50-TPβ receptor interaction is currently studied in our laboratory.

GTP-bound Goq preferentially activates PLC-β1 as described in the Introduction. Singer et al. (23) have shown that the GTP bound-Goq directly binds and activates PLC-β. Because on the one hand EBP50 inhibited the inositol phosphate production and on the other hand there had a greater affinity for the active form of Goq, the effect of EBP50 on the Goq-PLC-β1 interaction was investigated. We observed an almost complete inhibition in the interaction of Goq-R183C with PLC-β1 in the presence of EBP50. Taken together, the results described above indicate that EBP50 regulates the TP receptor signaling through Goq at different levels. First, we have shown that EBP50 interferes with the coupling of the TP receptors to Goq, by binding and preventing the GDP-bound Goq (inactive form) from binding to the TP receptor. Second, we have shown that EBP50 also interferes with the coupling of the activated Goq to the downstream effector PLC-β1.

GAPs regulate the heterotrimeric G protein signaling by increasing the rate of GTP hydrolysis by Go subunits up to 2000-fold. Both RGS proteins (regulators of G protein signaling) and G protein effectors (such as PLC-β and p115RhoGEF, which lack an RGS domain) belong to the family of GAP proteins. RGS proteins specifically bind the GTP-bound Go subunits and share an RGS domain involved in the regulation of the intrinsic GTPase activity of Go. Because our results have shown that EBP50 binds preferentially the GTP-bound Goq, and that this interaction is transient after activation of a GPCR, there was the possibility that EBP50 could modulate the intrinsic GTP hydrolysis activity of Goq. Analysis of the EBP50 amino acid sequence and its alignment with the RGS proteins failed to show any significant homology, indicating that EBP50 does not contain an RGS domain. Our results showed that EBP50 does not modulate the intrinsic GTPase activity of Goq. Regulation of Goq signaling by EBP50 must then rely principally on the mechanisms discussed above. We cannot, however, totally rule out the GAP activity of EBP50. Indeed, it was shown that the GRK2 GAP activity is apparent only in the presence of an activated GPCR (26), which suggests the possibility that receptors could have a critical role in potentiating GAP activity in cells. Moreover, it has been demonstrated that the ability of RGS2 to inhibit Goq-mediated signals in cells is highly dependent on the nature of the receptors that are being stimulated (32). The authors of this study suggested that regulatory selectivity may be conferred by specific receptor-RGS complexes. Thus, further investigation of the role of receptors in modulating EBP50-Goq interactions seem warranted.

EBP50 binds to NHE3 and is involved in its regulation by cAMP, as well as in its down-regulation (33). EBP50 may also be required to localize the H+ -ATPase in both apical and basolateral membranes in renal intercalated cells (33). Furthermore, EBP50 serves as a membrane retention signal for CFTR (34) and facilitates the dimerization of CPTFR that promotes the full expression of chloride channel activity (35). Moreover, recent experiments (6) have shown that EBP50 is involved in the dimerization of the platelet-derived growth factor receptor. Among its numerous functions, EBP50 also regulates the sorting of the β2-adrenergic receptor to either recycling endosomes or lysosomal degradatory pathways (36).
Since its isolation, EBP50 has been associated with a broad array of biological systems, all of which depend on the interaction of EBP50 through its PDZ1 and PDZ2 domains with the different target proteins. Our study identifies Goq as a novel interacting partner for the PDZ domains of EBP50. This finding is important in terms of G protein signaling regulation. The study of possible interactions between other PDZ domain-containing proteins with Go subunits constitutes a new and exciting field of research. It is also easy to envision the great impact of the Gαq-EBP50 interaction in signaling "cross-talk" because an EBP50 molecule bound to Gαq could possibly be unable to engage in the interaction with another binding partner.

indeed, one can imagine that multiple biological systems associated with EBP50 could be modulated by the activated Gαq-EBP50 interaction.

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