Phospholipase C-independent Activation of Glycogen Synthase Kinase-3β and C-terminal Src Kinase by Gαq

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It is generally thought that activation of phospholipase Cβ (PLCβ) by Gαq accounts for most of the effects of Gαq-coupled receptors. Here we describe a novel effect of Gαq, that is independent of the PLCβ pathway. Expression of the constitutively active Gαq mutant Gαq(Q209L) promoted an increase in glycogen synthase kinase-3β (GSK-3β) activity that was associated with increased phosphorylation of Tyr216 on GSK-3β, Gαq(Q209L)-AA, a mutant that cannot activate PLCβ, also induced GSK-3β activation and phosphorylation of Tyr216. We speculate that the protein-tyrosine kinase Csk (C-terminal Src kinase), which is also activated by Gαq(Q209L) and Gαq(Q209L)-AA, acts upstream of GSK-3β. Expression of Csk accentuated the activation of GSK-3β by Gαq(Q209L), whereas catalytically inactive Csk blocked Gαq(Q209L) activation by Gαq(Q209L). Recombinant Csk phosphorylated and activated GSK-3β in vitro, and GSK-3β coprecipitated with Csk from cell lysates. These results suggest that activation of Csk and GSK-3β by Gαq may contribute to the physiological and pathological effects of Gαq-coupled receptors.

The Gαq protein transduces signals from cell surface receptors that are activated by hormones such as angiotensin II, endothelin 1, catecholamines, and prostaglandin F2α to regulate diverse physiological functions. Activation of certain Gαq-coupled receptors appears to induce insulin resistance, because treatment of patients with receptor antagonists can improve insulin sensitivity (1, 2). In addition, persistent activation of Gαq is involved in the development of heart failure (3), which is one of the most common causes of death in humans. The most well characterized effector of Gαq is phospholipase Cβ (PLCβ), the activation of which leads to increased hydrolysis of phosphatidylinositol 4,5-bisphosphate, release of Ca2+ from intracellular stores, and activation of protein kinase C (4). It is generally thought that activation of this canonical PLCβ pathway by Gαq accounts for most of the pleiotropic effects of Gαq-coupled receptors (5).

Glycogen synthase kinase-3 (GSK-3) was discovered as a serine/threonine protein kinase that phosphorylates and inactivates glycogen synthase, but it is now known to regulate a diverse array of cellular processes (6, 7). Dysregulation of GSK-3 signaling is thought to play a role in the development of type II diabetes mellitus (8), neuronal cell loss in Alzheimer disease (9), and cancer (10). The two major isoforms of GSK-3 in mammalian tissues (GSK-3α and -3β) are structurally similar but not functionally equivalent. This was demonstrated upon deletion of the GSK-3β gene in mice, which resulted in embryonic lethality due to liver degeneration, even though GSK-3α was still presumably expressed at the normal level (11). GSK-3β activity is relatively high in resting cells, and it can be positively or negatively regulated by various stimuli. For example, insulin activation of the protein kinase Akt results in phosphorylation of GSK-3β at Ser9, thus reducing its enzymatic activity (12).

Several reports indicated that agonist stimulation of Gαq-coupled receptors antagonizes the signaling events initiated by insulin and other growth factors that lead to Akt activation (13–19). To directly investigate how Gαq affects Akt activity, we used Gαq(Q209L), a mutant that cannot hydrolyze GTP and therefore constitutively activates PLCβ. We found that Gαq(Q209L) blocks insulin and platelet-derived growth factor activation of Akt by inhibiting its upstream regulator phosphatidylinositol 3-kinase (PI3K) (20). Active Gαq still inhibited P13K/Akt in cells treated with U73122, a PLC inhibitor, suggesting that the inhibitory mechanism is independent of the canonical PLCβ pathway (20). In this study, we asked whether suppression of P13K/Akt signaling by activated Gαq results in increased GSK-3β activity. We show here that Gαq(Q209L) does induce the activation of GSK-3β, and use of a Gαq(Q209L) mutant that cannot activate PLC indicates that this response is independent of the canonical PLCβ pathway. Surprisingly, GSK-3β activation is not a consequence of decreased Akt activity but is instead because of activation of a tyrosine kinase. Finally, we identify Csk (C-terminal Src kinase), which has been shown to be activated by Gαq(Q209L) (21), as a tyrosine kinase that activates GSK-3β.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (3000 Ci/nmol) and myo-[3H]inositol (10–25 Ci/nmol) were from PerkinElmer Life Sciences. Ser(P)-9 GSK-3β antibody was from Cell Signaling Technology (Beverly, MA), and Tyr(P)-216 GSK-3β antibody was from BIOSOURCE (Camarillo, CA). GSK-3β antibody was from BD Biosciences (San Jose, CA). Antibodies to the hemagglutinin (HA) and Hisa epitopes were from Covance (Richmond, CA). Recombinant GSK-3β and Csk were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Lysates and Western Blotting—Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) with antibiotics and 10% horse serum (HyClone, Logan, UT). Cells at ~50% confluence were transfected using 3 μl of TransIT-293 (Mirus, Madison, WI) per μg of DNA. Equal amounts of each construct...
were used in cotransfections, except if otherwise noted. Cell lysates were prepared 2 days after transfection in lysis buffer containing 1% Triton X-100 as described earlier (20). Immunoprecipitation, Western blotting, and stripping of membranes were done as described elsewhere (17, 20).

**DNA Constructs**—Akt-HA was obtained from Richard Roth (Stanford University, Stanford, CA). The GSK-3β cDNA was isolated by reverse transcriptase-polymerase chain reaction using RNA from HEK 293 cells as template (19). (The RNA in Ref. 19 was erroneously reported to be from Swiss mouse 3T3 cells.) HA-GSK-3β and HA-GSK-3β 59A were described earlier (19). HA-GSK-3β Y216F was constructed using Pfu DNA polymerase (Stratagene) and the forward primer 5′-GAGGAGAAGAGGACACGCCTTTTATCGTTCGGATGACATAG. GSK-3α (Q209L) was described earlier (20). (The mouse GSK-α sequence we isolated shows two differences when compared with the published sequence for GSK-α from mouse brain (22). The C at position 84 in the coding region is G, and the G at position 85 is C. Amino acids 28 and 29 in the mouse GSK-α used here are thus Gln and Leu instead of His and Val. Others have reported a sequence identical to ours (23)). GSK-3β(Glu453Leu, His58Val) and Akt—were subcloned into pcDNA3.1 (Invitrogen). The cDNA for human Csk in pcDNA3.1/GS (tagged at the C terminus with the V5 epitope) Gsk-3 sequence we isolated shows Gln and Leu instead of His and Val. Others have reported a sequence identical to ours (23).) GSK-3β and Akt constructs were subcloned into pcDNA3.1 (Invitrogen). The DNA for human Csk in pcDNA3.1/GS (tagged at the C terminus with the V5 epitope and Hisα) was obtained from Invitrogen. Csk-KD was constructed using Pfu DNA polymerase and the forward primer 5′-CATGGAGGAGACACGACCTTTTATCGTTCGGATGACATAG to mutate Lys to Arg. The fidelity of all cDNA constructs was verified by sequencing.

**Kinase Assays**—GSK-3β activity was assayed using phospho-GS2 substrate peptide (Upstate Biotechnology) as described elsewhere (19). To assay Csk, equal amounts of protein in lysis buffer containing Triton X-100 were immunoprecipitated with His6 antibody and protein G-agarose at 4°C. The immunoprecipitates were washed twice with buffer A and twice with buffer B (50 mM HEPES, pH 7.4, 0.1 mM PMSF, 10 mM MgCl2, 1 mM MnCl2, and 1 mM sodium orthovanadate). Csk activity was measured in 20 μl of buffer A containing 0.25 μM substrate peptide (Upstate Biotechnology) and 1 μCi [γ-32P]ATP, 40 μM ATP, 1 μM dithiothreitol, and 5 μg/assay of poly(Glu, Tyr) (Sigma). After incubating the reactions at 30°C for 30 min, the mixtures were subjected to SDS-PAGE and autoradiography. Radioactive poly(Glu, Tyr) was cut out of the gel and counted in a scintillation counter.

**RESULTS**

**GSK-3β Activity**—In an earlier study we suggested that inhibition of PI3K/Akt by activated Gαq is independent of the canonical PLCβ pathway, based on its resistance to U73122 (20). Because U73122 is a relatively poor PLC inhibitor in our experimental systems, we decided to re-examine this issue using Gαq(Q209L)-AA, a Gαq mutant that still binds to GTP but does not activate PLC (mutant 10 cited in Ref. 24). Coexpression of Gαq(Q209L)-AA with HA-tagged Akt in HEK 293 cells caused a decrease in Akt activity that was comparable to that seen using Gαq(Q209L) (Fig. 1A). These results support our hypothesis that the inhibitory effect of active Gαq on Akt is independent of PLC activation.

GSK-3β activity is negatively regulated by Akt-mediated phosphorylation of Ser9 (25). We therefore expected that suppression of PI3K/Akt by Gαq(Q209L) would result in an increase in GSK-3β activity. This hypothesis was first tested using a stable cell line that expresses Gαq(Q209L) under the control of a doxycycline-inducible promoter (Flip-In T-Rex/293 cells) (20). These cells were transiently transfected with HA-tagged GSK-3β and then treated overnight with or without 1 μM doxycycline to induce expression of Gαq(Q209L). As expected, GSK-3β activity from cells treated with doxycycline was 1.8 times higher than the activity from vehicle-treated cells (p < 0.01, t test, n = 4). Doxycycline treatment of Flip-In T-Rex/293 cells that do not express Gαq(Q209L) did not affect GSK-3β activity. Similarly, cotransfection of HEK 293 cells with HA-GSK-3β and Gαq(Q209L) caused a 2.7-fold increase in GSK-3β activity as compared with the control cells (Fig. 1B). Gαq(Q209L)-AA stimulated GSK-3β to a comparable extent (Fig. 1B), indicating that activation of GSK-3β by Gαq(Q209L) is independent of the canonical PLCβ pathway. Control experiments confirmed that Gαq(Q209L)-AA does not activate PLC, as measured by accumulation of inositol phosphates, whereas Gαq(Q209L) causes a robust activation (Fig. 1C).

**GSK-3β Activity**—GSK-3β activity is negatively regulated by Akt-mediated phosphorylation of Ser9 (25). We therefore expected that suppression of PI3K/Akt by Gαq(Q209L) would result in an increase in GSK-3β activity. This hypothesis was first tested using a stable cell line that expresses Gαq(Q209L) under the control of a doxycycline-inducible promoter (Flip-In T-Rex/293 cells) (20). These cells were transiently transfected with HA-tagged GSK-3β and then treated overnight with or without 1 μM doxycycline to induce expression of Gαq(Q209L). As expected, GSK-3β activity from cells treated with doxycycline was 1.8 times higher than the activity from vehicle-treated cells (p < 0.01, t test, n = 4). Doxycycline treatment of Flip-In T-Rex/293 cells that do not express Gαq(Q209L) did not affect GSK-3β activity. Similarly, cotransfection of HEK 293 cells with HA-GSK-3β and Gαq(Q209L) caused a 2.7-fold increase in GSK-3β activity as compared with the control cells (Fig. 1B). Gαq(Q209L)-AA stimulated GSK-3β to a comparable extent (Fig. 1B), indicating that activation of GSK-3β by Gαq(Q209L) is independent of the canonical PLCβ pathway. Control experiments confirmed that Gαq(Q209L)-AA does not activate PLC, as measured by accumulation of inositol phosphates, whereas Gαq(Q209L) causes a robust activation (Fig. 1C).
strated by Western blotting of cell lysates using the HA antibody (Fig. 2A, bottom panel).

To further examine the relationship between increased phosphorylation of Tyr216 and GSK-3β activation, we tested whether Goq(Q209L) can still activate a GSK-3β mutant in which Tyr216 is changed to Phe (HA-GSK-3β Y216F). The enzymatic activity of HA-GSK-3β Y216F was 20% that of wild type HA-GSK-3β (28), and cotransfection with Goq(Q209L) did not increase its activity (Fig. 2B). In contrast, a GSK-3β mutant with Ala substituted for Ser9 (HA-GSK-3β S9A) had high basal activity as compared with wild type HA-GSK-3β (19), and the presence of Goq(Q209L) caused an additional 20% increase in its activity (Fig. 2B). These results support the hypothesis that Goq(Q209L)-dependent activation of GSK-3β is mediated by phosphorylation of Tyr216.

Csk Activates GSK-3β in Vivo—It was recently reported that expression of Goq(Q209L) causes an increase in the activity of Csk (21), a ubiquitously expressed protein-tyrosine kinase that phosphorylates Src family members to negatively regulate their activity (29, 30). Therefore, we wondered if Csk might mediate Goq(Q209L)-induced phosphorylation of GSK-3β. We confirmed that Csk is activated in HEK 293 cells upon coexpression of Goq(Q209L) (Fig. 3). Goq(Q209L-AA) was as effective as Goq(Q209L) in activating Csk (3.3-fold stimulation), indicating that this response is also independent of PLC activation (Fig. 3).

Next, we tested whether Goq(Q209L) activation of Csk leads to increased GSK-3β activity in vivo. Coexpression of HA-GSK-3β with either Csk or Goq(Q209L) alone increased GSK-3β activity 2- to 2.8-fold, respectively (Fig. 4A). However, GSK-3β activation increased to 4.9-fold in the presence of both Csk and Goq(Q209L) (Fig. 4A). By contrast, expression of catalytically inactive Csk-KD did not increase GSK-3β activity (Fig. 4B). More importantly, overexpression of Csk-KD blocked the Goq(Q209L)-induced activation of GSK-3β (Fig. 4B). Csk-KD most likely acts as a dominant-negative suppressor of endogenous Csk, which activates GSK-3β in the presence of Goq(Q209L).

Csk Phosphorylates and Activates GSK-3β in Vitro—The results above raised the possibility that Csk might directly activate GSK-3β by phosphorylating Tyr216. To test whether Csk can phosphorylate GSK-3β in vitro, purified recombinant Csk was incubated with purified recombinant GSK-3β in the presence of [γ-32P]ATP and LiCl to inhibit GSK-3β autophosphorylation. We found that GSK-3β was phosphorylated by Csk
Fig. 5. Csk phosphorylates and activates GSK-3β in vitro. A. 2 ng of purified recombinant GSK-3β was incubated at 37 °C for 1 h with or without 50 ng of purified recombinant Csk in 15 μl of buffer B (50 mM HEPES, pH 7.4, 100 μM EDTA, 10 mM MgCl₂, 1 mM dithiothreitol, 100 μM sodium orthovanadate, and 100 μM ATP) plus 100 mM LiCl and 5 μCi of [γ-32P]ATP. The reaction mixtures were separated by SDS-PAGE followed by autoradiography to visualize GSK-3β. B, 2 ng of recombinant GSK-3β was preincubated at 30 °C with or without 100 ng of recombinant Csk in 10 μl of buffer B plus 0.1 mg/ml bovine serum albumin. After 30 min, 1 μg of phospho-GS2 substrate peptide and 2.5 μCi of [γ-32P]ATP in 5 μl of buffer B were added, and GSK-3β activity was assayed. The experiment was repeated three times with similar results. C, HEK 293 cells were transfected with the indicated constructs. Cell lysates were made in buffer containing Nonidet P-40 plus sodium deoxycholate (20) without EDTA. Proteins pulled down with nickel-nitrotriacetic acid (Ni²⁺-NTA) agarose (Qiagen) were examined on a Western blot probed with HA antibody (top panel). A Western blot of cell lysate proteins probed with antibodies to HA and His₆ (6xHis) shows that HA-GSK-3β and Csk-His₆ (Csk-6xHis) were expressed appropriately (lower two panels).

DISCUSSION

Accumulating evidence indicates that PLCβ is not the only effector of Goq. Direct binding of Goq to Bruton’s tyrosine kinase (Btk) has been shown to increase Btk kinase activity (31). We found that activated Goq inhibits PI3K/Akt signaling via an inhibitor mechanism that is independent of PLC activation and that might involve an inhibitory interaction between Goq and p110α PI3K (Fig. 1A and Ref. 20). The results presented here suggest that the activation of Csk and subsequent tyrosine phosphorylation and activation of GSK-3β represents another novel effector pathway for Goq, that functions independently of PLC. An effector pathway with similar characteristics was described in 3T3L1 adipocytes, where Goq(Q209L) stimulated the translocation of the GLUT4 glucose transporter in a manner that was independent of PLC activation but blocked by tyrosine kinase inhibitors (32).

Goq(Q209L)-AA, in which Arg²⁵⁶ and Thr²⁵⁷ are changed to Ala, was originally made to identify residues in Goq that are required for interaction with PLC (24). We show here that although Goq(Q209L)-AA is almost completely defective in the ability to activate PLC, it is indistinguishable from Goq(Q209L) in the ability to inhibit Akt and activate GSK-3β and Csk. The simplest interpretation of these results is that these effects of Goq(Q209L) are independent of PLC activation. In contrast to our results, it was recently reported that the ability of Goq(Q209L) to depress PI3K/Akt activity in cardiomyocytes requires PLC activation (33). This conclusion was based on the use of a Goq(Q209L) mutant with three Ala substitutions that cannot activate PLC (mutant 7 cited in Ref. 24). An alternative explanation for the inability of this mutant to inhibit Akt is that it lacks the ability to bind to and inhibit p110α PI3K.

In light of the inhibitory effect of Goq(Q209L) on Akt, we were surprised to find that Goq(Q209L) activates GSK-3β by causing an increase in Tyr²¹⁶ phosphorylation, instead of a decrease in Ser⁹ phosphorylation. Indeed, treatment of cells with LY 294002, a PI3K inhibitor, did not activate HA-GSK-3β or HA-GSK-3β and Csk interact in vivo. HA-GSK-3β and His₆-tagged Csk were expressed in cells, and Csk was pulled down using a Ni²⁺ affinity resin. HA-GSK-3β coprecipitated with Csk from lysates of cells expressing both proteins (Fig. 5C). These data support the hypothesis that GSK-3β is an in vivo substrate of Csk.

(Fig. 5A). To determine whether phosphorylation by Csk changes GSK-3β activity, the two enzymes were incubated separately or together in the presence of ATP prior to assaying GSK-3β activity. GSK-3β activity was increased 3-fold after incubation with Csk (Fig. 5B). Finally, we tested whether GSK-3β and Csk interact in vivo. HA-GSK-3β and His₆-tagged Csk were expressed in cells, and Csk was pulled down using a Ni²⁺ affinity resin. HA-GSK-3β coprecipitated with Csk from lysates of cells expressing both proteins (Fig. 5C). These data support the hypothesis that GSK-3β is an in vivo substrate of Csk.
activate the enzyme by causing an increase in Tyr216 phosphorylation (27, 34). LPA signals through G protein-coupled receptors that can couple to Goq, Goq, and Goq12/13. Sayas et al. (27) showed that expression of activated mutants of Goq12 and Goq13 mimicked the effect of LPA by inducing GSK-3 activation. Goq4 was not tested because treatment of cells with a PLC inhibitor did not block LPA activation of the kinase, apparently ruling out Goq as a possible mediator of LPA-induced GSK-3 activation (27). Our finding that Goq(Q209L) activates GSK-3 independently of PLC raises the possibility that Goq4 might contribute to the activating effect of LPA on GSK-3.

We considered several candidate tyrosine kinases that might activate GSK-3 in response to Goq(Q209L). The most well characterized GSK-3 tyrosine kinase is Zak1, which controls cell fate specification in Dictyostelium (35, 36). Binding of cAMP to the CAR3 receptor activates Zak1, which phosphorylates GSK-3 on tyrosine residues (including the Tyr216 equivalent) to increase its activity. A mammalian homolog of Zak1 has not been identified. The Src family tyrosine kinase Fyn has been shown to phosphorylate recombinant GSK-3β in vitro (37). However, we found that treatment of cells with PP2, an inhibitor of Src family kinases, did not block the activation of GSK-3β by Goq(Q209L).2 The Ca2+-dependent tyrosine kinase PYK2 (proline-rich protein kinase 2) has also been shown to phosphorylate GSK-3β in vitro and has been proposed to mediate increased tyrosine phosphorylation of GSK-3β in response to transient increases in intracellular Ca2+ (38). Our finding that Goqα(Q209L)-AA activates GSK-3β without activating PLC implies that an increase in intracellular Ca2+ and PYK2 activation are not required for this response. Finally, as mentioned above, active Goq binds to Btk and stimulates its kinase activity (31). Because Btk expression is limited to a subset of hematopoietic cells, we did not consider this kinase as a likely candidate to activate GSK-3β in HEK 293 cells.

It was reported that Csk activity is increased upon expression of Gbγ heterodimers and activated forms of Goqα, Goq12, and Goq13 (21). Interestingly, as mentioned above, activated mutants of Goq12 and Goq13 also activate GSK-3 (27). These observations led us to explore whether Goqα(Q209L), Csk, and GSK-3β act within the same signaling pathway. Our data are consistent with the hypothesis that GSK-3β and Csk act between Goqα(Q209L) and GSK-3β. Both kinases are activated by Goqα(Q209L)-AA as well as by Goqα(Q209L) expression of Csk activates GSK-3β, expression of Goqα(Q209L) augments the activation of GSK-3β by Csk, and Csk-KD inhibits Goqα(Q209L)-mediated GSK-3β activation. In addition, our results showing that Csk phosphorylates and activates GSK-3β in vitro and that the two kinases interact with each other in cell lysates suggest that GSK-3β is a direct target of Csk. We speculate that activation of GSK-3β by Goqα12 and Goqα13 is also mediated by Csk (21, 27). The mechanism for Csk activation by Goqα(Q209L) is not understood. Gbγ, but not the activated mutants of Goqα, Goq12, or Goq13, bound directly to Csk and increased its tyrosine kinase activity in vitro (21). Expression of Gbγ and the activated Goa subunits caused Csk to translocate from the cytoplasm to the membrane, but it is not known how this might result in Csk activation (21).

Recent studies suggest that activation of GSK-3β by tyrosine phosphorylation is an important mechanism by which apoptotic stimuli can lead to cell death. Nerve growth factor withdrawal or staurosporine treatment of cultured neuronal cells caused an increase in GSK-3β activity, Tyr216 phosphorylation, and cell death (26). Similar observations were made using in vivo models of neuronal apoptosis induced by ischemia or blockade of N-methyl-D-aspartate receptors (26, 39). Activated Goa also causes apoptosis in some cell types (40). In addition, transgenic mice overexpressing Goa die of heart failure, and cardiomyocytes from these animals show an increased rate of apoptosis (41). The apoptotic effect of Goα might be mediated in part by activation of GSK-3β. It is also well established that GSK-3β affects glucose metabolism by phosphorylating and inactivating glycogen synthase (6). In the liver, hormone stimulation of some Goα-coupled receptors also inhibits glycogen synthesis (42). This phenomenon could be partly because of activation of GSK-3β by Goq. Further studies are needed to elucidate how Goα activation of Csk and GSK-3β might contribute to the regulation of apoptosis, glucose metabolism, and other cellular effects of Goα-coupled receptors.