G13α-mediated PYK2 Activation

PYK2 IS A MEDIATOR OF G13α-INDUCED SERUM RESPONSE ELEMENT-DEPENDENT TRANSCRIPTION*

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G12α/G13α transduces signals from G-protein-coupled receptors to stimulate growth-promoting pathways and the early response gene c-fos. Within the c-fos promoter lies a key regulatory site, the serum response element (SRE). Here we show a critical role for the tyrosine kinase PYK2 in muscarinic receptor type 1 and G12α/G13α signaling to an SRE reporter gene. A kinase-inactive form of PYK2 (PYK2 KD) inhibits muscarinic receptor type 1 signaling to the SRE and PYK2 itself triggers SRE reporter gene activation through a RhoA-dependent pathway. Placing PYK2 downstream of G-protein activation but upstream of RhoA, the expression of PYK2 KD blocks the activation of an SRE reporter gene by GTPase-deficient forms of G12α or G13α but not by RhoA. The GTPase-deficient form of G12α triggers PYK2 tyrosine phosphorylation, and co-expression of the RGS domain of p115 Rho-GEF inhibits both responses. Finally, we show that in vivo G13α, although not G12α, readily associates with PYK2. Thus, G-protein-coupled receptors via G13α activation can use PYK2 to link to SRE-dependent gene expression.

Growth factors rapidly induce the c-fos gene, a response that depends upon a critical cis regulatory site located in the c-fos promoter termed the serum response element (SRE).1 Two transcriptional factors bind the SRE, the 67-kDa serum response factor (SRF), which binds as a dimer to the CarG box of the SRE, and the ternary complex factor (TCF), which binds SRF along with a purine-rich sequence at the 5′-end of the SRE (1). TCF, which is encoded by several ets-related genes including Elk-1 and SRF accessory proteins 1 and 2, can only bind the SRE in conjunction with SRF. TCF phosphorylation enhances ternary complex formation and its transcriptional activity. At least three major mitogen-activated kinases including ERK1/2, p38, and stress-activated protein kinase (SAPK, also referred to as Jun kinase or JNK) phosphorylate TCF (2–4). A mutant SRE that lacks the TCF binding site no longer responds to activators of these kinase pathways but remains responsive to stimuli that activate SRF (5, 6).

Agonists of some G-protein-coupled receptors (GPCRs) selectively activate SRF without significant TCF activation (5). GPCR-induced SRF activation requires RhoA (5). Heterotrimeric G-proteins of the G12/G13 class directly stimulate Rho guanine nucleotide exchange factors (GEFs) and thereby trigger RhoA activation (7, 8). In NIH 3T3 cells, GTPase-deficient G12α/G13α potently triggers SRF-dependent gene transcription, and RhoA inhibitors efficiently block it (9). The following findings implicate the Tec/Bmx nonreceptor tyrosine kinase family in the regulation of RhoA and SRF by G12α/G13α: kinase-defective Bmx inhibits SRF activation by GTPase-deficient forms of G12α/G13α, GTPase-deficient G12α/G13α stimulates Tec kinase activity, and Tec kinases synergize with GTPase-deficient G12α/G13α to activate SRF via RhoA (10).

Agonists of some GPCRs also activate the tyrosine kinase PYK2 (11–14). GPCRs that couple to Gα subfamily members stimulate phospholipase C-β, which causes the release of intracellular calcium and protein kinase C activation and a subsequent enhancement of PYK2 kinase activity (13, 15). Downstream of PYK2 signaling lie the p38, SAPK, and Erk pathways, each of which can lead to TCF phosphorylation (11, 16–18). In addition, PYK2 triggers c-src activation (11), which can stimulate SRF-dependent SRE activation (10). Thus, in cell types that express PYK2, GPCR signaling by triggering PYK2 activation could enhance SRF-mediated transcription through TCF-dependent and -independent pathways. To examine this possibility, we used HeLa cells, a cell line that contains significant endogenous levels of PYK2. Surprisingly we found a requirement for PYK2 in SRF activation triggered by engagement of the M1R or by expression of GTPase-deficient G12α and G13α. In the course of these studies, we observed that GTP-G13α potently stimulates PYK2 kinase activity and that it associates with PYK2 in vivo.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Expression vectors for the GαQ204L (QL), GαQ226L (QL), GαQ209L (QL), GαQ229L (QL), GαQ226L (QL), RhoAQ63L (QL), β1, 7c SRE-L-CAT, M1R; and the C3 exotoxin were kindly provided by Dr. S. Gutkind (National Institutes of Health). The Myc-PYK2 expression vector was kindly provided by Dr. S. Earp (University of North Carolina). The coding region for the Rho GDP-dissociation inhibitor (19) was isolated by PCR from a universal cDNA library (CLONTECH, Palo Alto, CA) and subcloned into pCRIII. The pSRE-luciferase plasmid was purchased (Stratagene, La Jolla, CA). Full-length p115 RhoGEF was subcloned into pCANTat using the EcoRI and XhoI sites and kindly provided by Dr. M. Hart (Oxyx Pharmaceuticals, Richmond, CA; Ref. 7). A PCR product prepared from pCANTat p115RhoGEF, which encodes the RGS domain (amino acids 1–252), kindly provided by Dr. P. C. Sternweis (University of Texas Southwestern Medical Center, Dallas, TX), was inserted into pCMV5 to

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1 The abbreviations used are: SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor; SAPK, stress-activated protein kinase; GPCR, G-protein-coupled receptor; GEF, guanine nucleotide exchange factor; GDI, GDP dissociation inhibitor; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; RBD, Rho binding domain; CAT, chloramphenicol acetyltransferase; HA, hemagglutinin.
create pCMV5-p115 RGS. pGEX-2T containing the Rhoetkin Rho binding domain was kindly provided by Dr. M. A. Schwartz ( Scripps Research Institute, La Jolla, CA). The antibodies against the following were purchased: PYK2 and Rho (Transduction Laboratories, San Diego, CA); G12a, G13a, G12a, YQL and Rho (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The co-immunoprecipitations were performed using lysates (20 μg Tris, pH 8.0, 137 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium azide in TTBS overnight. The blots were washed twice with TTBS before the addition of a biotinylated goat anti-rabbit immunoglobulin (DAKO, Carpinteria, CA) diluted 1:1500 in TTBS containing 5% fetal calf serum. Following a 1-h incubation, the blot was washed twice with TTBS and then incubated with streptavidin conjugated to horseradish peroxidase (DAKO). The signal was detected by enhanced chemiluminescence (ECL) following the recommendations of the manufacturer (Amersham Pharmacia Biotech).

G12a/13a-mediated PYK2 Activation

To determine which activated G-proteins enhance SRE-dependent gene expression in HeLa cells, we transfected constructs that directed the expression of GTPase-deficient forms of Ga or β1 and γ2 along with a reporter gene that contains five copies of the SRE and a minimal promoter fused to luciferase. Similar to the results reported with NIH 3T3 cells (9), GTPase-deficient G12a and G13a potently induced SRE-luciferase activity (Fig. 1A). G12aQL and G13aQL both increased the reporter gene activity more than 200-fold compared with the basal activity. In contrast, GαQQL induced only a 10-fold increase, and the other stimuli had minimal effects, yet GαQQL and GαQQL substantially elevated the intracellular levels of inositol phosphates and cAMP, respectively, and GαQQL reduced intracellular levels of cAMP (data not shown).

PYK2 expression also activated the SRE-luciferase reporter construct. While not as efficient as the GTPase deficient forms of G12a or G13a, PYK2 induced a 20–25-fold increase in SRE-luciferase activity (Fig. 1B). Signaling to the SRF by activators of heterotrimeric G-proteins requires RhoA, while c-src triggers SRF activation through a RhoA-independent pathway (10). To determine whether PYK2 induces SRE reporter gene activation by activating RhoA, we simultaneously expressed the botulinum C3 exoenzyme. The toxin ADP-ribosylates RhoA at asparagine 41, thereby deactivating it and preventing the activation of downstream effectors (5). Surprisingly, the co-expression of the C3 toxin markedly impaired the ability of PYK2 to activate the SRE reporter construct, indicating a requirement for Rho activation (Fig. 1B).

This result suggested that PYK2 activates the SRE through an SRF-dependent mechanism, a possibility we tested using a TCF-insensitive SRE-CAT construct (5). TCF cannot bind because of a mutation in the TCF binding site (pSREEmutL). PYK2 readily activated this construct (Fig. 1C). Furthermore, C3 toxin and a specific RhoA inhibitor, which inhibits the dissociation of GDP from RhoA (RhoGDI) (19), both inhibited the activation. As expected, both C3 toxin and RhoGDI blocked SRF activation through a RhoA-independent pathway (10). To determine whether PYK2 induces SRE reporter gene activation by activating RhoA, we simultaneously expressed the botulinum C3 exoenzyme. The toxin ADP-ribosylates RhoA at asparagine 41, thereby deactivating it and preventing the activation of downstream effectors (5). Surprisingly, the co-expression of the C3 toxin markedly impaired the ability of PYK2 to activate the SRE reporter construct, indicating a requirement for Rho activation (Fig. 1B).

In Vitro Kinase Assay—PYK2-immunoprecipitates were washed three times with kinase lysis buffer (20 mM Hepes pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, 1 mM Na3VO4, and 10% glycerol) with protease inhibitors. After these washes and six additional washes (three with a LiCl wash buffer (500 mM LiCl, 100 mM Tris, pH 7.4, 0.1% Triton X-100, and 1 mM diothithreitol) and three with kinase reaction buffer (30 mM HEPES, pH 7.5, 10 mM MgCl2, and 2 mM MnCl2)), the kinase assays were performed. poly(Glu, Tyr) (4:1) (Sigma) was used as a substrate.

Rhoa Translocation—The Rhoa translocation experiment was done by a modification of a previously published procedure (10). Briefly, COS-7 cells transfected with PYK2 or PYK2 KD expression vector were incubated in a hypotonic buffer containing 20 mM Tris and protease inhibitors for 30 min on ice, lysed by several cycles of freezing and thawing, and sonicated for five 10-s intervals. To make the membrane preparations, the samples were centrifuged at 3000 × g for 5 min, and the supernatant was recentrifuged at 52,000 rpm for 30 min at 4 °C. The membrane pellets were washed three times with the hypotonic buffer. The sample buffer contained 50 mM Hepes, 150 mM NaCl, 20 mM Tris, pH 7.4, 15% glycerol, 1 mM dithiothreitol, and 1% Triton X-100. The samples were precipitated with 6% (m/v) trichloroacetic acid and 0.0125% (m/v) deoxycholate. Equal amount of proteins from the membrane and the supernatant were loaded onto SDS-PAGE, and the proteins were detected by the PYK2- and RhoA-specific antibodies, respectively.

Rho Binding Assay—Bacterially expressed GST-Rhotekin Rho binding domain protein (GST-RBD) was purified from isopropyl-1-thio-β-D-
did the C3 toxin. A similar analysis of M1R signaling to the SRE revealed a similar inhibition (Fig. 2B). To verify that M1R signaling triggered the SRE reporter through a G12/G13-linked signaling pathway in HeLa cells, we repeated the M1R signaling experiments in the presence of the RGS domain of p115 RhoGEF (p115 RGS), a GTPase-activating protein for G12α and G13α (7). The expression of p115 RGS nicely blocked the activation of the SRE reporter construct induced by signaling through the M1R (Fig. 2C). These data indicate that GPCRs, which couple to G12α and G13α, can activate SRF-dependent transcription through a PYK2-dependent pathway.

G13αQL and to a Lesser Degree G12αQL Activate PYK2—While M1R signaling leads to PYK2 tyrosine phosphorylation and enhanced PYK2 kinase activity (13), the effects of G12α/G13 on PYK2 have not been previously reported. To examine their effects, we transfected the PYK2 expression vector in the presence or absence of constructs that directed the expression of G12αQL or G13αQL and subjected immunoprecipitated PYK2 to an in vitro kinase using an exogenous substrate, poly(Glu, Tyr) (4:1). Both G12αQL and G13αQL enhanced PYK2 kinase activity toward the exogenous substrate; however, G13αQL induced a higher level (Fig. 3A). We also noted a phosphorylated band that migrated at a rate identical to that of PYK2 in the lysates from stimulated cells. To directly determine if the stimuli triggered PYK2 tyrosine phosphorylation, we analyzed the PYK2 immunoprecipitates by immunoblotting with an anti-phosphotyrosine antibody. While G12αQL and M1R signaling enhanced PYK2 tyrosine phosphorylation, G13αQL had a very modest effect. The expression of G12αQL or G13αQL did not alter PYK2 expression levels. By immunoblotting with PYK2 phosphotyrosine-specific antibodies (Fig. 3B), we discovered that G12αQL led to the phosphorylation of PYK2 on residues Tyr402, a major autophosphorylation site and site of interaction with Src (11); Tyr779 and Tyr881 in the kinase domain; and Tyr881 in the proline-rich region. In contrast, G13α failed to induce high levels of reactivity with the antibodies. These results suggest that while both G12αQL and G13αQL activate PYK2, G13αQL is more efficient.

In HeLa Cells, PYK2 Acts Predominantly Upstream of RhoA and Downstream of G12/G13—The inhibition of M1R, G12αQL, and G13αQL triggered SRE reporter gene activity by PYK2 KD did not delineate where in the signaling pathway PYK2 functions. However, the dependence of PYK2-induced SRE reporter gene activity upon RhoA activation implied a role for PYK2 upstream of RhoA. To test that prediction, we examined whether the PYK2 KD construct inhibited RhoA-induced SRE reporter gene activity. We found that the same concentration of PYK2 KD that inhibited G1αQL-induced SRE reporter gene activity by 75% only reduced RhoA-induced reporter gene activity by 10–20% (Fig. 4A). Furthermore, G13αQL enhanced PYK2 kinase activity independent of RhoA activation, because C3 toxin co-expression had no effect on the PYK2 kinase activity (data not shown).

Since a tyrosine phosphorylation event precedes the translocation of RhoA to the plasma membrane (29), PYK2 expression could alter the intracellular location of RhoA (Fig. 4B). Indeed, the expression of PYK2 but not PYK2 KD in COS-7 cells enhanced the amount of endogenous RhoA found in a crude membrane fraction. In contrast, neither the wild type PYK2 nor the kinase defective form altered the amount of G1α in the membrane preparations. This led us to examine whether PYK2 expression increased the amount of activated Rho in HeLa cells. To do so, we used the RBD of Rhotekin to affinity-precipitate GTP-Rho (21) from HeLa cells lysates expressing RhoA and PYK2 (Fig. 4C). As controls, we performed similar experiments with PYK2 KD or p115 RhoGEF. We found that PYK2 KD did not alter the basal amount of affinity-precipitated GTP-Rho; however, both wild type PYK2 and the positive control, p115 RhoGEF, increased the amount. Thus, the ex-

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**Fig. 1.** G12α, G13α, and PYK2 induce SRE reporter gene activity through a RhoA- and SRF-dependent pathway. A, test of various stimuli on the induction of the SRE-reporter. HeLa cells were transfected with the constructs directing the expression of the indicated G-proteins (0.5 μg) in the presence of an SRE-luciferase reporter gene (0.1 μg). Data are shown as -fold induction compared with basal and are the mean and S.D. of three experiments. B, PYK2 expression triggered the SRE reporter. HeLa cells were transfected with either of two concentrations of PYK2 expression vector (1 and 2 μg) with SRE-luciferase in the presence or absence of the C3 toxin reporter construct (0.25 μg). Data are shown as -fold induction compared with basal and are the mean and S.D. of three experiments. PYK2 levels were detected by immunoblotting cell lysates. C, PYK2 triggered the SRF-dependent reporter through a RhoA-dependent pathway. HeLa cells were transfected with constructs directing expression of PYK2 (1.5 μg) and SRE_L-CAT (0.1 μg) in the presence or absence of C3 toxin (0.2 or 0.4 μg) or a RhoA inhibitor (0.2 or 0.4 μg). Data are shown as relative CAT activity compared with basal and are the mean ± S.D. of two experiments. D, inhibition of p115 RhoGEF-induced SRE reporter activity by C3 toxin or a RhoA inhibitor. HeLa cells were transfected with constructs directing expression of p115 RhoGEF (0.5 μg) and SRE-luciferase (0.1 μg) in the presence or absence of C3 toxin (0.2 or 0.4 μg) or RhoA inhibitor (0.2 or 0.4 μg). Data are shown as relative luciferase activity compared with basal. An immunoblot of p115 RhoGEF (Myc) is shown.
pression of PYK2 in HeLa cells results in the translocation and the activation of a portion of the cellular pool of RhoA.

Finally, to verify the specificity of p115 RGS and to analyze the effect of RhoA on PYK2 activation, we expressed GTPase-deficient forms of G12a, G13a, and RhoA, and M1R in the presence of PYK2 and p115 RGS or not. By binding to GTP-G12a and GTP-G13a, p115 RGS should inhibit their ability to stimulate downstream effectors (7). We found that co-expression of p115 RGS blocked PYK2 activation by GTPase-deficient forms of G12a and G13a but not by GTPase-deficient G12a (Fig. 5). Suggesting that in HeLa cells G13a/G12a activation was important for M1R signaling to PYK2, p115 RGS markedly inhibited PYK2 activation. Consistent with PYK2 acting predomi-

Fig. 2. PYK2 KD inhibits G13aQL-, G12aQL-, and carbachol-induced SRE reporter gene activity. A, PYK2 KD inhibited G13aQL- and G12aQL-induced SRE reporter gene activity. HeLa cells transfected with constructs directing expression of G13aQL (0.5 μg) or G12aQL (0.5 μg) in the presence or absence of PYK2 KD (0.5 or 1 μg) or C3 toxin (0.25 μg) were assessed for SRE-luciferase activity. The levels of the expressed proteins were determined by immunoblotting. Data are from three experiments. B, PYK2 KD inhibited carbachol signaling through the M1R to the SRE reporter. SRE-luciferase activity was detected in HeLa cells expressing M1R (0.5 μg), PYK2 KD (0.5 or 1 μg), and C3 (0.25 μg) as indicated. The cells were exposed to carbachol (100 μM) for the last 5 h of the culture. Data are from two experiments. C, p115 RGS blocks carbachol signaling through the M1R to the SRE reporter. SRE-luciferase activity was detected in HeLa cells expressing M1R (0.2 μg) and p115 RGS (0.5, 1.0, or 1.5 μg) or not. The cells were exposed to carbachol (100 μM) for the last 5 h of the culture. Data are representative of two experiments performed.

Fig. 3. G13aQL potently induces PYK2 kinase activity and PYK2 tyrosine phosphorylation. A, effect of G13aQL, G12aQL, and carbachol on PYK2 kinase activity and PYK2 tyrosine phosphorylation. The PYK2 kinase assay used PYK2 immunoprecipitated from cells expressing G13aQL (0.25 μg), M1R (0.5 μg), or G12aQL (0.25 μg). The M1R transfected cells were exposed to carbachol (100 μM) for 15 min prior to the assay. First panel, autoradiograph of kinase assay in the presence of substrate; second panel, autoradiograph of kinase assay in the absence of substrate, band with the mobility of PYK2 indicated; third panel, phosphotyrosine immunoblot (Western blot, W.B.) of immunoprecipitated PYK2; fourth panel, PYK2 immunoblot; fifth panel, G13a immunoblot; sixth panel, G13a immunoblot, seventh panel, PYK2 tyrosine phosphorylation using Tyapt402-, Tyapt579-, Tyapt580-, or Tyapt881-specific antibodies. PYK2 was detected by phosphotyrosine-specific antibodies in lysates prepared from HeLa cells expressing G13aQL or G12aQL or not.

Fig. 3. G13αQ potently induces PYK2 kinase activity and PYK2 tyrosine phosphorylation. A, effect of G13αQ, G13αQ, and carbachol on PYK2 kinase activity and PYK2 tyrosine phosphorylation. The PYK2 kinase assay used PYK2 immunoprecipitated from cells expressing G13αQ (0.25 μg), M1R (0.5 μg), or G13αQ (0.25 μg). The M1R transfected cells were exposed to carbachol (100 μM) for 15 min prior to the assay. First panel, autoradiograph of kinase assay in the presence of substrate; second panel, autoradiograph of kinase assay in the absence of substrate, band with the mobility of PYK2 indicated; third panel, phosphotyrosine immunoblot (Western blot, W.B.) of immunoprecipitated PYK2; fourth panel, PYK2 immunoblot; fifth panel, G13αQ immunoblot; sixth panel, G13αQ immunoblot; seventh panel, PYK2 tyrosine phosphorylation using Tyapt402-, Tyapt579-, Tyapt580-, or Tyapt881-specific antibodies. PYK2 was detected by phosphotyrosine-specific antibodies in lysates prepared from HeLa cells expressing G13αQ or G12αQ or not.

Finally, to verify the specificity of p115 RGS and to analyze the effect of RhoA on PYK2 activation, we expressed GTPase-deficient forms of G12a, G13a, RhoA, and M1R in the presence of PYK2 and p115 RGS or not. By binding to GTP-G12a and GTP-G13a, p115 RGS should inhibit their ability to stimulate downstream effectors (7). We found that co-expression of p115 RGS blocked PYK2 activation by GTPase-deficient forms of G12a and G13a but not by GTPase-deficient G12a (Fig. 5). Suggesting that in HeLa cells G13a/G12a activation was important for M1R signaling to PYK2, p115 RGS markedly inhibited PYK2 activation. Consistent with PYK2 acting predomi-

G13α and PYK2 Associate in Vivo—The activation of G13α could either directly or indirectly lead to PYK2 activation. If G13α directly stimulated PYK2, we should be able to detect an interaction between the two proteins. To test whether the two proteins interact, we co-transfected HeLa cells with G13αQL and PYK2. G13αQL was epitope-tagged with HA, which was useful for immunoprecipitating the protein but not for immunoblotting. We analyzed either HA or FLAG (control) antibody immunoprecipitates for the presence of PYK2 using a Myc tag-specific antibody, which recognized Myc-tagged PYK2 (Fig. 6A). We found that the HA-G13αQL immunoprecipitates contained readily detectable PYK2, while the control FLAG immunoprecipitates did not. In similar experiments using HA-tagged G12αQL and PYK-2, the two proteins did not co-immunoprecipitate. We confirmed the presence of G12αQL and G13αQL in the immunoprecipitates by immunoblotting with G-protein specific antibodies. Conversely, we examined Myc-Pyk2 immunoprecipitates for the presence of G13αQL or G12αQL via immunoblotting with G-protein-specific antibodies (Fig. 6B). Again, G12αQL readily co-immunoprecipitated with PYK2, while G13αQL did not.

These results indicate that GTP-G12αQ can associate in vivo with PYK2. To provide better evidence that this is the case, we determined whether G-protein activation leads to a detectable
G₁₃α-PYK2 complex in nontransfected HeLa cells. We used a nonspecific activator of heterotrimeric G-proteins, treatment with aluminum fluoride, to stimulate endogenous heterotrimeric G-proteins and then examined PYK2 immunoprecipitates for the presence of G₁₃α by immunoblotting (Fig. 6C). We found a clear band reactive with the G₁₃α antisera, which migrated with the appropriate molecular mass when we used cells that had been treated with aluminum fluoride for 30 min prior to lysis. Control immunoprecipitates failed to contain any such band. Thus, the activation of G₁₃α probably leads to its transient association with PYK2 in PYK2-expressing cells.

DISCUSSION

Three sets of observations support our conclusion that G₁₃α can use PYK2 to link to downstream signaling pathways. First, in HeLa cells, overexpression of either PYK2 or G₁₃αQL activates SRF through a RhoA-dependent mechanism, and a kinase-inactive form of PYK2 blocks G₁₃αQL from inducing SRE-dependent transcription. This inhibition appears specific, since the kinase-active PYK2 does not block the triggering of SRE-dependent transcription by RhoA. Second, expression of GTPase-deficient G₁₃α activates PYK2 as assessed by an in vitro kinase assay using a known PYK2 substrate and by immunoblotting with phosphorylation state-specific antibodies. The latter verifies that G₁₃α drives tyrosine phosphorylation of the major PYK2 autophosphorylation site. Furthermore, the G₁₃α domain of p115 RGS impairs PYK2 activation by either M₁R or G₁₃αQL. Third, GTP-G₁₃αQL and PYK2 interact. In cells expressing PYK2 and G₁₃αQL, the two proteins readily co-immunoprecipitate. In contrast, a similar interaction did not occur when we substituted G₁₁αQL for G₁₃αQL. Confirming that a physiologically relevant G₁₁α/PYK2 interaction occurs, the activation of endogenous heterotrimeric G-proteins leads to a rapid association between G₁₁α and PYK2. While the easy detection of G₁₁α with PYK2 indicates that PYK2 could be a direct G₁₁α effector, the activation of PYK2 may be secondary to other signaling pathways that G₁₁α stimulates. We are currently mapping the regions in PYK2 necessary to detect the interaction with G₁₁α.

We also find that G₁₂αQL expression activates PYK2 and that PYK2 KD interferes with the activity of an SRE reporter following G₁₂αQL expression in HeLa cells. G₁₂αQL induces a very high level of SRE reporter gene activity even greater than that we observe with G₁₁αQL. However, an interaction between G₁₁αQL and PYK2 sufficient to allow the proteins to co-immunoprecipitate does not occur, suggesting a fundamental difference in how G₁₁α and G₁₂α activate PYK2. Further studies will be needed to illuminate how G₁₁α and G₁₂α activate PYK2.

A recent study implicates Tec/Bmx nonreceptor tyrosine kinases in the regulation of Rho and serum response factor by G₁₃αQL. Since a kinase defective Bmx more readily impairs SRF activation by G₁₃αQL, G₁₁αQL is likely the major protein to mediate this activation. This implies that the activation of SRF by G₁₃αQL is intrinsic to the cell and not dependent on the activation of a downstream effector.
are immunoblotted for PYK2 (left panel). Activation, Tec kinase or PYK2 overexpression enhances SREB protein expression is shown in the cell lysates. Precipitation; Ab. Levels are shown in the cell lysate. Western blot; I.P.

**Fig. 6.** PYK2 interacts with G\(_{13}a\). A. PYK2 immunoprecipitated with G\(_{13}a\)-QPL. HeLa cells were co-transfected with constructs directing the expression of Myc-PYK2 (1 \(\mu\)g) and HA-G\(_{13}a\)-QPL (1 \(\mu\)g) or HA-G\(_{13}a\)-QPL (1 \(\mu\)g). HA (G-protein) or FLAG (control) immunoprecipitates were immunoblotted for Myc (PYK2), G\(_{13}a\), or G\(_{13}a\). PYK2 and G-protein expression is shown in the cell lysates. B. G\(_{13}a\)-QPL immunoprecipitated with PYK2. Similar experiment as shown in A except that Myc (PYK2) or FLAG (control) immunoprecipitates were immunoblotted for Myc (PYK2), G\(_{13}a\), or G\(_{13}a\). C. Endogenous PYK2 and G\(_{13}a\) co-immunoprecipitated following G-protein activation. HeLa cells were treated with AlF\(_4\) or not prior to lysis. PYK2 or control immunoprecipitates were immunoblotted for PYK2 (left panel) or G\(_{13}a\) (right panel). PYK2 levels are shown in the cell lysate. W.B., Western blot; I.P., immunoprecipitation; Ab., antibody.

press varying amounts of the Tec kinases and PYK2, in some cell types G\(_{13}a\) may largely use PYK2 and in others it may use Tec kinases to activate SRF, whereas in some cells it may use both kinases. Arguing that both kinases may contribute to SRF activation, Tec kinase or PYK2 overexpression enhances SRE reporter activity significantly less than does GTPase-deficient G\(_{13}a\)/G\(_{13}a\). To examine the potential for Tec kinases to activate the SRF in HeLa cells, we have tested whether Bruton’s tyrosine kinase (BTK) enhances transcription of the SRE-luciferase reporter. Arguing that expression of BTK should be functionally equivalent to the expression of other Tec kinases, Bmx, Txk, Tec, and Itk all reconstitute BTK signaling in cell lines deficient for BTK (24). We find that a constitutively active form of BTK induces only a 2–3-fold increase in reporter gene activity, while wild type PYK2 induces a 20-fold enhancement; however, the combination of PYK2 and BTK leads to a 60-fold increase. Indicating that is not downstream of BTK, the expression of PYK2 KD does not block SRE activation by BTK.

How do Tec kinases and PYK2 lead to Rho activation and hence the SRF? The basal activity of Tec kinase and PYK2 in NIH 3T3 and HeLa cells leads to SRF activation in the respective cell type (Ref. 10 and our data). In both instances, the activation of SRF requires functional kinase domains and Rho activation, implying the need for the phosphorylation of downstream effectors and for GTP-Rho. Both Tec kinase and PYK2 enhance the translocation of Rho to COS-7 cell membranes, results consistent with the inhibition of Rho translocation by tyrosine kinase inhibitors (23). Tec overexpression induces cytoskeletal reorganization in NIH 3T3 cells (10), and PYK2 overexpression increases the amount of GTP-Rho in HeLa cells. Thus, both Tec kinase and PYK2 can cause Rho activation. RhoGEFs, RhoGDIs, and RhoGAPs control Rho activity (GTP-versus GDP-bound state) by triggering the exchange of GTP for GDP, inhibiting the release of GDP from RhoA, or by enhancing the rate that GTP-Rho hydrolyzes GTP back to GDP, respectively (reviewed in Ref. 25). G\(_{13}a\) itself can stimulate Rho by activating RhoGEFs. Tec kinases and PYK2 or one of their targets could regulate Rho activity by potentiating G\(_{13}a\)’s activation of the exchange factors. While the Tec TH domain binds to VAV, a hematopoietic specific exchange factor for RhoA, Rac, and Cdc42 (25), no evidence currently links PYK2 to RhoGEFs. Further studies will determine whether PYK2 enhances their activity. Alternatively, PYK2 or Tec kinase could either directly or indirectly deactivate RhoGDIs or RhoGAPs, which would enhance the likelihood of RhoA being GTP-bound and able to activate downstream effectors.

Besides involvement in SRF activation by G\(_{13}a\) in some cell types, PYK2 could also participate in G\(_{13}a\)’s induction of SAPKs. G\(_{12}a\), G\(_{13}a\), and G\(_{13}a\) activate SAPK via different mechanisms depending upon the cell type. In COS-7 cells, they activate SAPK through Cdc42, while in NIH 3T3 cells (26) and COS-1 cells Ras is critical (27). In HEK 293 cells, a dominant negative form of RhoA and C3 exoenzyme blocks G\(_{13}a\)-mediated SAPK activation as does C-terminal Src kinase (Csk), suggesting the additional involvement of a Csk (28). In NIH 3T3 cells, a dominant negative Ras inhibits G\(_{12}a\)-mediated SAPK activation, but it fails to do so in HEK 293 cells, where a dominant negative form of Rac does so (29). These differences probably arise because of the differing availability of downstream mediators of G\(_{12}a\) and G\(_{13}a\) signaling. In those cells that express PYK2, we would expect that G\(_{13}a\) activation should lead to PYK2 and Src kinase activation, both of which can contribute to SAPK activation.

Activation of certain heterotrimeric G-proteins causes neurite retraction and cell rounding through a RhoA-dependent pathway (30). In PC12 cells, the induction of neurite retraction and cell rounding by GTPase-deficient G\(_{12}a\), G\(_{13}a\), or G\(_{13}a\) also depends upon RhoA (31). The tyrosine kinase inhibitor tyrphostin A25 blocks the morphological changes triggered by GTPase-deficient G\(_{13}a\) or G\(_{13}a\) but not by G\(_{12}a\), while the inhibition of protein kinase C or elimination of extracellular calcium inhibits the morphological changes induced by GTPase-deficient G\(_{13}a\) but not by G\(_{12}a\) or G\(_{13}a\) (32). Furthermore, lysophosphatidic acid induces neurite retraction through a G\(_{13}a\)-initiated pathway that requires tyrosine kinase activity (33). Since PC12 and neuronal cells express high levels of PYK2 (32), its activation by G\(_{13}a\) via the release of intracellular calcium or by G\(_{13}a\) through a yet unknown mechanism could account for these

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C-S. Shi, unpublished observations.
results. Finally, in PC12 cells transfected with the α-1A adrenergic receptor, norepinephrine activates mitogen-activated protein kinase pathways and triggers PYK2 tyrosine phosphorylation independent of calcium release or protein kinase C activation (34). These results suggest that either GTP-Gq can stimulate PYK2 through a pathway not dependent upon calcium release or protein kinase C or that the α-1A adrenergic receptor uses G13α/G13α to activate PYK2.

Pozzo to receive activation signals from GPCRs both via the activation of Gqα and G13α and located at a critical juncture in many signaling pathways, PYK2 processes upstream information to coordinate the activation of downstream signaling pathways, which alter the activity of transcription factors such as SRF and TCF. Interestingly, also subject to many of the same ways, which alter the activity of transcription factors such as SRF and TCF. Interestingly, also subject to many of the same downstream signaling pathways that many signaling pathways, PYK2 processes upstream information to coordinate the activation of downstream signaling pathways, which alter the activity of transcription factors such as SRF and TCF. Interestingly, also subject to many of the same downstream signaling pathways that many signaling pathways, PYK2 processes upstream information to coordinate the activation of downstream signaling pathways, which alter the activity of transcription factors such as SRF and TCF. Interestingly, also subject to many of the same downstream signaling pathways that many signaling pathways, PYK2 processes upstream information to coordinate the activation of downstream signaling pathways, which alter the activity of transcription factors such as SRF and TCF. Interestingly, also subject to many of the same downstream signaling pathways that many signaling pathways, PYK2 processe