The inhibitory γ subunit of the type 6 retinal cGMP phosphodiesterase functions to link c-Src and G-protein-coupled receptor kinase 2 in a signaling unit that regulates p42/p44 mitogen-activated protein kinase by epidermal growth factor.*

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The inhibitory γ subunit of the retinal photoreceptor type 6 cGMP phosphodiesterase (PDEγ) is phosphorylated by G-protein-coupled receptor kinase 2 on threonine 62 and regulates the epidermal growth factor-dependent stimulation of p42/p44 mitogen-activated protein kinase in human embryonic kidney 293 cells. We report here that PDEγ is in a pre-formed complex with c-Src and that stimulation of cells with epidermal growth factor promotes the association of GRK2 with this complex. c-Src has a critical role in the stimulation of the p42/p44 mitogen-activated protein kinase cascade by epidermal growth factor, because c-Src inhibitors block the activation of this kinase by the growth factor. Mutation of Thr-62 (to Ala) in PDEγ produced a GRK2 phosphorylation-resistant mutant that was less effective in associating with GRK2 in response to epidermal growth factor and did not potentiate the stimulation of p42/p44 mitogen-activated protein kinase by this growth factor. The transcript for a short splice variant version of PDEγ lacking the Thr-62 phosphorylation site is also expressed in certain mammalian cells and, in common with the Thr-62 mutant, failed to potentiate the stimulatory effect of epidermal growth factor on p42/p44 mitogen-activated protein kinase. The mutation of Thr-22 (to Ala) in PDEγ, which is a site for phosphorylation by p42/p44 mitogen-activated protein kinase, resulted in a prolonged activation of p42/p44 mitogen-activated protein kinase by epidermal growth factor, suggesting a role for this phosphorylation event in the negative feedback control of PDEγ.

Mitogenic stimuli initiate cell proliferation via different classes of cell surface receptors that include growth factor receptor tyrosine kinase receptors and G-protein-coupled receptors (GPCRs).† This involves stimulation of the p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) pathway (1, 2). In certain cases, growth factor- and GPCR agonist-mediated stimulation of the p42/p44 MAPK pathway require the G-protein-regulated aggregation of signaling molecules followed by endocytosis of receptor signal complexes at clathrin-coated pits via a dynamin II-dependent process (3). For instance, isoproterenol, insulin-like growth factor-1, platelet-derived growth factor, fibroblast growth factor, and nerve growth factor can sometimes use G-proteins, G-protein-coupled receptor kinase 2 (GRK2), and β-arrestin I/Ii to regulate the p42/p44 MAPK pathway (4–11). GRK2 is activated by G-protein βγ subunits and phosphorylates GPCRs, which are, in certain cases, associated with growth factor receptors (10, 12). The phosphorylation of the GPCR promotes binding of β-arrestin, which is required for dynamin II-dependent endocytosis of the receptor-signal complex and subsequent activation of p42/p44 MAPK (13). Thus, the expression of dominant-inhibitory β-arrestin I or dynamin II mutants impairs insulin-like growth factor-1, β-adrenergic-, and lysophosphatic acid-dependent activation of p42/p44 MAPK (14–16). In the presence of these inhibitory mutants, the p42/p44 MAPK signaling cascade proceeds only as far as Raf activation (16). β-Arrestin is a clathrin adaptor that binds certain receptor complexes and targets them to clathrin-coated pits, whereas dynamin II is a GTPase involved in the “pinching off” of clathrin-coated endocytic vesicles containing the receptor-signal complex (17). The hydrolysis of GTP by dynamin II is believed to be catalytic for pinching off of endocytic vesicles and subsequent recollection of receptor signal complexes with cytoplasmic MEK-1 and p42/p44 MAPK.

The phototransduction cascade involving rhodopsin (GPCR), GRK, β-arrestin, and RGS9β5 (18) bears many similarities with signaling by growth factors and G-protein-coupled receptors in other mammalian cell systems. The phototransduction cascade involves cGMP phosphodiesterases that are expressed in rod and cone photoreceptors (termed PDE6) as tetrameric proteins composed of two catalytic subunits and two γ subunits (PDEγ). PDEγ inhibits cGMP hydrolysis at the catalytic sites. The two types of photoreceptor cells, rod and cone, express different isoforms of PDEγ. These proteins differ in their extreme N-terminal regions, whereas the central polycationic and C-terminal domains that are involved in the interaction with both PDE6 and transducin are almost identical.

We have found that PDEγ has a wider role in mammalian cell biology (19–22). Indeed, we have reported that rod PDEγ is expressed in lung, kidney, testes, liver, heart, airway, and pulmonary smooth muscle and HEK 293 cells and is absent from these tissues in rod PDEγ knockout mice. We have also identified a novel role for PDEγ in regulating the EGF- and...
thrombin-dependent activation of the p42/p44 MAPK pathway in HEK 293 cells (21). We also found that GRK2 is required for the stimulatory effect of rod PDEγ on both the EGF- and thrombin-dependent activation of p42/p44 MAPK. Indeed, rod and cone PDEγ are substrates for phosphorylation by GRK2. Moreover, a GRK2 phosphorylation-resistant (Thr-62 changed to Ala) rod PDEγ mutant failed to increase the EGF- or thrombin-dependent activation of p42/p44 MAPK, and in fact functioned as a dominant negative. We also presented evidence to show that thrombin stimulates the formation of a complex between rod PDEγ and dynamin II (21). This is significant because it is well established that GTP hydrolysis by dynamin II promotes endocytosis of vesicles containing receptor signal complexes that subsequently relocalize with and activate cytoplasmic p42/p44 MAPK. Taken together, the data are consistent with the phosphorylation of Thr-62 in rod PDEγ by GRK2 being essential for interaction with dynamin II.

In this paper, we have further explored the dynamic of the interaction between PDEγ and GRK2. We show for the first time that PDEγ is a functional linker/regulator of both cell culture and GRK2. We also show that a GRK2 phosphorylation-resistant PDEγ mutant (Thr-62 PDEγ mutant) is less effective than the wild type protein in binding GRK2 in response to EGF. The mutant appears to function as an endogenous dominant negative by acting as a sink for c-Src. We also provide the first evidence for a negative feedback mechanism involving p42/p44 MAPK that regulates PDEγ and appears to limit the duration of p42/p44 MAPK activation in response to EGFR.

**EXPERIMENTAL PROCEDURES**

**Materials**—All biochemicals were from Roche Applied Science, whereas general chemicals were from Sigma. Cell culture supplies were from Invitrogen. Anti-phospho-p42/p44 MAPK and anti-dynamin II antibodies were from New England Biolabs. Anti-Grb-2 and anti-p42/p44 MAPK antibodies were from Transduction Laboratories (Lexington, KY). Anti-Src and anti-GRK2 antibodies were from Santa Cruz Biotechnology. pRK5-GRK2 cDNA plasmid construct was a kind gift from Professor R. Lefkowitz (Duke University). Anti-PDEγ antibody to the C-terminal domain of photoreceptor PDEγ and which reacts with both rod and cone isoforms was a kind gift from Dr. R. Cote (University of New Hampshire).

**Cell Culture**—HEK 293 cells were maintained in minimal essential medium containing 10% (v/v) fetal calf serum. These cells were placed in minimal essential medium for 24 h before experimentation. ASM cells were maintained in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal calf serum and 10% donor horse serum. These cells were incubated on an end-over-end shaker for 24 h at 37°C. The cell lysate supernatant was removed from the monolayer cell and washed with ice-cold phosphate-buffered saline and lysed in 1 ml of buffer containing 1× phosphate-buffered saline, 1% (v/v) Nonidet P-40, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 1 mM sodium orthovanadate, 2 mM PMSF, leupeptin, aprotinin (all protease inhibitors were removed from the lysates) were passed through the 21-gauge needle to shear the DNA. The lysates were then incubated at 4°C for 30 min. The cell lysate supernatant was then harvested by centrifugation at 10,000 x g for 10 min at 4°C.

**Recombinant His-tagged Rod PDEγ**—The open reading frame of mouse lung rod PDEγ was subcloned into an expression vector pTrcHis-B (Invitrogen). The vector was transformed into TOP10F E. coli strain. The E. coli was grown in 2 ml of Luria Broth (LB) containing 10% (w/v) tryptone, 5% (w/v) yeast extract, and 10% (w/v) NaCl supplemented with 50 μg/ml ampicillin at 37°C overnight. The overnight E. coli culture was then diluted 50-fold with 100 ml of LB medium containing 50 μg/ml ampicillin and grown for an additional 2.5 h, until the absorbance of the culture was 0.6–0.8. Isopropyl-β-D-thiogalactoside was then added to a final concentration of 1 mM for induction of the culture at 30°C. After a suitable period of induction (3 h), the cells were harvested by centrifugation (A-18C rotor Centrifrik 4-22K 15 min at 7,400 rpm at 4°C) and resuspended in lysis buffer containing 20 mM NaH2PO4, pH 7.8. Each sample was then lysed by 5 times ultrasonication followed by 3 times freeze-thaw cycles in lysis buffer. All buffers above contained protease inhibitors (20 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM benzamidine, and 0.1 mM PMSF) to inhibit proteolytic reaction in the sample. The lysed cells were then centrifuged at 13,000 rpm/10 min at 4°C (Sigma laboratory centrifuge IK15) to remove the insoluble cell debris and the resulting supernatant was stored at −70°C.

**Magnetocapture Assay**—Ni-NTA magnetic agaroose beads (Qiagen) were resuspended by vortexing for 2 s. 500 μl of the recombinant His-tagged rod PDEγ was immediately added to 50 μl of the 5% (w/v) Ni-NTA magnetic agaroose beads suspension. The suspension was incubated on an end-over-end shaker for 1 h at 4°C. This was to allow efficient binding of the His-tagged rod PDEγ to the Ni-NTA magnetic agaroose beads. After 1 h, the microcentrifuge tubes containing the complexes were placed on a 12-tube magnetic separator for 1 min, and the supernatant was removed with a pipette. The magnetic beads-His-tagged rod PDEγ complexes were washed with wash buffer (50 mM NaH2PO4, 300 mM NaCl, and 20 mM imidazole, pH 8.0) for 3 times at 4°C. After the wash buffer was removed by placing the magnetic beads-microcentrifuge tubes on the magnetic separator for 1 min. The HEK 293 cell lysate supernatant was then added to the magnetic beads. The suspension was incubated on an end-over-end shaker for 1 h at 4°C. After 1 h, the complexes were washed with wash buffer once, and the wash buffer was removed as described above. The potential interaction proteins with His-tagged rod PDEγ were eluted with elution buffer containing 50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0, and collected for detection by SDS-PAGE/Western blot.

**Immunoprecipitation Assay**—The medium was removed, and cells were lysed in ice-cold lysis buffer (1 ml containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1% (v/v) Nonidet P-40, 1% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) SDS, 0.5% (w/v) glycine, 1% (v/v) Triton X-100, 0.5% (w/v) sodium orthovanadate, 0.2% PMSF, leupeptin, pepstatin, and aprotinin (all protease inhibitors were at 10 μg/ml, pH 8.0)) for immunoprecipitation. The cells were harvested and centrifuged at 13,000 rpm for 5 min at 4°C. The concentration of cell lysate supernatant was determined and equalized by performing performing a Bradford colorimetric assay (0.5 mg/ml). Cell lysate supernatant was electrophoresed in polyacrylamide gel as positive control.

For immunoprecipitation assay, cell lysate supernatant (500 μl) was taken for immunoprecipitation with specific antibodies (2 μg of antibodies and 100 μl of 1:1 protein A-Sepharose CL4B; 1:1 indicates equal part of protein A-Sepharose and immunoprecipitation buffer). After 4°C incubation of 1 h at 2 h and 4°C, the mixture was centrifuged at 13,000 rpm for 15 s at 4°C. Immunoprecipitates were washed twice with ice-cold buffer A (containing 10 mM Hepes, pH 7.0, 100 mM NaCl, 0.2 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 0.5% (v/v) Nonidet P-40) and once in buffer B without Nonidet P-40.
**RESULTS AND DISCUSSION**

**Interaction between PDEγ, GRK2, and c-Src**—The ability of GRK2 to phosphorylate PDEγ in an EGF-dependent manner prompted us to investigate whether these proteins exist in a complex in HEK 293 cells. In addition, several reports (23) have shown that growth factors activate c-Src and GRK2 and that these proteins regulate each other in a bidirectional manner. Moreover, in previous studies (21) we reported that the overexpression of GRK2 and/or PDEγ increases the activation of p42/p44 MAPK induced by EGF. Therefore, HEK 293 cells were transfected with PDEγ plasmid constructs and PDEγ, c-Src, and GRK2 immunoprecipitated with specific respective antibodies from lysates of cells treated with and without EGF. We report here that PDEγ and c-Src exist in a pre-formed complex and that stimulation of cells with EGF promotes association of GRK2 with the PDEγ-c-Src complex. This was based upon several lines of evidence. First, Fig. 1a shows that recombinant overexpressed PDEγ (14 kDa) and c-Src (60 kDa) are co-immunoprecipitated with GRK2 (85 kDa) from lysates of HEK 293 cells using anti-GRK2 antibodies. The amount of both c-Src and recombinant PDEγ co-immunoprecipitated with GRK2 was increased from cells treated with EGF (fold stimulations in response to EGF: c-Src, 2.74 ± 1.26-fold; PDEγ, 1.75 ± 0.05-fold, n = 3, p < 0.05 versus control). The EGF-dependent increase in the amount of c-Src and PDEγ retrieved in anti-GRK2 immunoprecipitates is entirely to be expected if c-Src and PDEγ are indeed pre-complexed, and EGF stimulation of cells promotes their association with GRK2. The data obtained are therefore entirely compatible with this model. Second, Fig. 1b shows that PDEγ and GRK2 were co-immunoprecipitated with c-Src using anti-c-Src antibodies. The amount of PDEγ co-immunoprecipitated was not increased from cells treated with EGF. In contrast, the amount of GRK2 associated with PDEγ decreased from control treated with EGF (fold stimulations in response to EGF: GRK2, 1.85 ± 0.05-fold; PDEγ, 1.75 ± 0.05-fold, n = 3, p < 0.05 versus control). The EGF-dependent increase in the amount of GRK2 expressed in cells overexpressing PDEγ compared with cells expressing only endogenous PDEγ. The fact that the association of PDEγ with c-Src is not sensitive to EGF stimulation provides additional evidence that these

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**Fig. 1. Interactions of rod PDEγ with GRK2, c-Src, and Grb2 in HEK 293 cells.** HEK 293 cells were transiently transfected with vector, pRK5-GRK2, or rod PDEγ pcDNA-6xHis plasmid constructs. The cells were then stimulated with EGF (50 ng/ml) for 5 min. a, Western blots (WB) showing co-immunoprecipitation of rod PDEγ and c-Src with GRK2 from control and EGF-stimulated transfected cells using anti-GRK2 antibodies (Ab). b, co-immunoprecipitation of GRK2, rod PDEγ, and Grb2 with c-Src from control and EGF-stimulated transfected cells using anti-c-Src antibodies. c, co-immunoprecipitation of p42/p44 MAPK with PDEγ using anti-c-Src antibodies. d, reverse-transfected cells with rod PDEγ from control and EGF-stimulated transfected cells using anti-C-terminal PDEγ antibodies. e, HEK 293 cell lysates (CL) were used as positive control for the various proteins and run on the same SDS-PAGE as the immunoprecipitates (IP). f, Western blots showing the effect of recombinant rod PDEγ and GRK2 on the EGF-dependent activation of p42/p44 MAPK in transfected cells. g, Western blots showing the effect of PP2 on the EGF-dependent activation of p42/p44 MAPK in vector- and rod PDEγ-transfected cells. The cells were pretreated with c-Src inhibitor, PP2 (10 μM, 15 min). h, Western blots showing the effect of recombinant rod PDEγ and GRK2 on the EGF-dependent activation of p42/p44 MAPK in vector- and rod PDEγ-transfected cells. The cells were pretreated with c-Src inhibitor, PP2 (10 μM, 15 min).
proteins are in a pre-formed complex that is strictly EGF-independent. The increased amount of GRK2 in anti-c-Src immunoprecipitates isolated from cells treated with EGF provides additional evidence that it is the binding of GRK2 to the PDEγ-c-Src complex that is, in fact, EGF-dependent. We also found that Grb-2 (26 kDa) was co-immunoprecipitated with c-Src, indicating association between these proteins. This association was also increased in cells transfected with PDEγ. Third, Fig. 1c shows that c-Src and GRK2 were co-immunoprecipitated with PDEγ from lysates. The amount of c-Src co-immunoprecipitated with PDEγ using anti-PDEγ antibodies was not increased from cells treated with EGF. Again, this is entirely in line with our interpretation that the c-Src-PDEγ complex is pre-formed in an EGF-independent manner. The amount of GRK2 co-immunoprecipitated with PDEγ was increased from cells treated with EGF (fold stimulations in response to EGF: GRK2, 2.01 ± 0.82-fold, n = 3, p < 0.05 versus control), again indicating that it is the GRK2 binding step that is EGF-dependent. None of the proteins were co-immunoprecipitated when antibodies were omitted from the immunoprecipitation procedure (data not shown).

Only a small fraction of PDEγ is present in the complex with c-Src and GRK2 (see Fig. 1, a and b compared with c). Thus, only a limited increase in the expression of this protein is actually required to bind c-Src and GRK2 in order to support EGF receptor signaling. This is corroborated by previous findings (21) showing that PDEγ is limiting for EGF receptor signaling to p42/p44 MAPK. The current findings are also compatible with the fact that cells are stimulated with a single agonist that might use only a relatively small fraction of the PDEγ expressed. The fold increases in the EGF-dependent association of GRK2 with the c-Src-PDEγ complex reported here are also in agreement with the potentiation of EGF-stimulated p42/p44 MAPK activation by PDEγ (see Fig. 1d). In addition to the recombinant form of the protein, we have also reported previously (21) that endogenous PDEγ participates in regulating EGF receptor signaling to p42/p44 MAPK. This was supported by data showing that the effect of endogenous PDEγ can be ablated by transfection of cells with PDEγ antisense plasmid construct (21). Endogenous PDEγ, c-Src, and GRK2 clearly form a complex in cells, as significant amounts of these proteins were isolated in anti-Src immunoprecipitates from vector-transfected cells (Fig. 1b). As with overexpressed recombinant PDEγ, EGF promoted the association of GRK2 with endogenous PDEγ in this complex (Fig. 1b).

Our findings are important because they are the first to define a role for PDEγ as a functional linker between GRK2 and c-Src. Thus, PDEγ may function to recruit GRK2 close to c-Src, whereupon there may be the reciprocal activation of each kinase. This functional interaction between PDEγ, GRK2, and c-Src is important because the formation of the complex appears to be required for EGF-dependent activation of p42/p44 MAPK. Thus, overexpression of PDEγ or GRK2 increased EGF-dependent activation of p42/p44 MAPK (Fig. 1d), fold stimulations in response to EGF versus vector-transfected cells: rod PDEγ, 1.7 ± 0.1; GRK2, 1.8 ± 0.2, n = 3, p < 0.05 versus vector-transfected cells), whereas pretreatment of cells with the c-Src inhibitor, PP2, ablated EGF-dependent activation of p42/p44 MAPK (Fig. 1e). We have also obtained additional evidence for a mechanistic link between c-Src-PDEγ and p42/p44 MAPK. We report here that the p42/p44 MAPK activated in response to EGF associates with the c-Src-PDEγ complex. Thus, phosphorylated p42/p44 MAPK is present in anti-PDEγ immunoprecipitates with PDEγ, c-Src, and GRK2 from vector- and PDEγ-transfected cells treated with EGF but not from...
Recombinant Histagged bacterially expressed rod PDEγ was immobilized on Ni-NTA magnetic agarose beads by preincubating the bacterial lysates with magnetic beads. HEK 293 cells were treated with or without EGF (50 ng/ml) for 5 min. HEK 293 cell lysates were then incubated with Ni-NTA magnetic agarose beads-His-tagged rod PDEγ. HEK 293 cell lysates (both control and EGF-stimulated) were separated by SDS-PAGE and serve as positive control to determine the migration of specific proteins. The protein complexes were eluted with 250 mM imidazole elution buffer.

Western blotting (WB) of imidazole eluates with antibodies. These are representative results of an experiment performed three times.

Interaction of c-Src, GRK2, and p42/p44 MAPK with PDEγ

**Fig. 4.** The EGF-dependent effect on the interaction between immobilized PDEγ and PDEγ-dynam II-GRK2-c-Src-Grb2 complexes. Recombinant Histagged bacterially expressed rod PDEγ was immobilized on Ni-NTA magnetic agarose beads by preincubating the bacterial lysates with magnetic beads. HEK 293 cells were treated with or without EGF (50 ng/ml) for 5 min. HEK 293 cell lysates were then incubated with Ni-NTA magnetic agarose beads-His-tagged rod PDEγ. HEK 293 cell lysates (both control and EGF-stimulated) were separated by SDS-PAGE and serve as positive control to determine the migration of specific proteins. The protein complexes were eluted with 250 mM imidazole elution buffer. a, Western blotting (WB) of imidazole eluates with antibodies (Ab) to dynamin II, GRK2, c-Src, and Grb2; b, Western blotting of imidazole eluates with anti-C-terminal rod PDEγ antibodies. These are representative results of an experiment performed three times.

**Fig. 5.** The Thr-22 mutant PDEγ functions to prolong EGF-dependent activation of p42/p44 MAPK in airway smooth muscle cells. ASM cells were transiently transfected with vector (H) or rod PDEγ and/or Thr-22 mutant rod PDEγ pcDNA-3.1 plasmid constructs. The cells were then stimulated with EGF (50 ng/ml) for the indicated times. Western blots showing the time course of EGF-stimulated p42/p44 MAPK activation in vector or rod PDEγ- or Thr-22 mutant rod PDEγ-transfected cells. p42/p44 MAPK activation was detected using anti-phospho-p42/p44 MAPK specific antibodies. Blots were also stripped and re-probed with anti-p42 MAPK antibodies to ensure equal protein loading. These are representative results of an experiment performed three times.

control cells (Fig. 1f). These data strongly suggest that the inhibition of the pool of PDEγ associated c-Src by PP2 is directly responsible for the attenuation of the EGF-dependent activation of p42/p44 MAPK by this compound. The results therefore further highlight the physiological significance of the PDEγ-c-Src complex in regulating this kinase pathway. These findings also suggest that the PDEγ-c-Src-GRK2 complex might undergo endocytosis and relocalization with components of the p42/p44 MAPK pathway.

Role of Thr-62 in PDEγ Interaction with c-Src and GRK2—In previous studies we reported that GRK2 phosphorylates PDEγ on Thr-62. Thus, mutagenesis of the Thr-62 (to Ala) produces a protein whose phosphorylation by GRK2 is severely impeded (21). Mutagenesis of the Thr-62 in PDEγ has no impact on the folding of this protein, which exists in solution as a polypeptide without tertiary structure. In contrast with wild type PDEγ, the Thr-62 mutant cannot support EGF-dependent activation of p42/p44 MAPK and, indeed, functions as a dominant negative to block the involvement of endogenous PDEγ in regulating p42/p44 MAPK signaling (Fig. 2a, fold stimulations of p42/p44 MAPK in response to EGF versus vector-transfected cells: rod PDEγ, 1.6 ± 0.34; Thr-62 PDEγ, 0.52 ± 0.02-fold, n = 3, p < 0.05 versus vector-transfected cells). We now show that the mutant PDEγ is not an efficient binding partner of GRK2 in cells stimulated with EGF when compared with wild type PDEγ (Fig. 2b). Consistent with this, we found that the amount of GRK2 associated with c-Src was reduced by 52 ± 30% (n = 3, p < 0.05 versus vector-transfected cells) in EGF-stimulated cells overexpressing Thr-62 mutant PDEγ compared with vector-transfected cells. This finding indicates that the mutant might prevent GRK2 binding to endogenous PDEγ (Fig. 2b). How can this be achieved? One possibility is that the Thr-62 mutant might act as a sink for c-Src, thereby preventing interaction of c-Src with GRK2 via endogenous PDEγ. Consistent with this possibility is our finding that the Thr-62 mutant was still capable of binding c-Src. Thus, the amount of wild type or Thr-62 mutant PDEγ co-immunoprecipitated with c-Src was similar (Fig. 2b). Mutation of Thr-62 in PDEγ also reduced the interaction between the c-Src and Grb-2 (Fig. 2b).

Short Cone PDEγ—We have also detected transcript for a short cone PDEγ isoform, which has a 41-bp deletion (corresponding to exon 3) resulting in a frame change (22). This deletion produces a new “in-frame” stop codon resulting in an early termination to produce a truncated protein (short cone PDEγ) that lacks Thr-62. This protein is predicted to have an identical N-terminal and polycationic mid-region but a different C-terminal domain compared with the larger version of cone PDEγ.

We have therefore investigated the effect of the truncated recombinant cone PDEγ on p42/p44 MAPK signaling. Overexpression of the truncated cone PDEγ reduced the EGF-dependent activation of p42/p44 MAPK (Fig. 3, upper panel, fold stimulations of p42/p44 MAPK in response to EGF versus vector-transfected cells: rod PDEγ, 1.6 ± 0.34; short cone PDEγ, 0.59 ± 0.27; rod PDEγ plus short cone PDEγ, 1.15 ± 0.06, n = 3–6, p < 0.05 for rod PDEγ plus short cone PDEγ-transported versus rod PDEγ-transfected cells).

The increase in thrombin-dependent activation of p42/p44 MAPK induced by either rod or large cone PDEγ was also reduced in cells overexpressing the truncated cone PDEγ (Fig. 3, lower panel, fold stimulations of p42/p44 MAPK in response to thrombin versus vector-transfected cells: rod PDEγ, 2.3 ± 0.2; cone PDEγ, 2.05 ± 0.18; short cone PDEγ, 0.77 ± 0.15; short cone PDEγ plus rod PDEγ, 0.95 ± 0.03; short cone PDEγ plus short cone PDEγ, 0.84 ± 0.12, n = 3–6, p < 0.05 for rod or cone PDEγ plus short cone PDEγ-transported versus rod or cone PDEγ-transfected cells). Thus, the truncated cone PDEγ may also act as an endogenous dominant negative modulator of EGF- and thrombin-dependent stimulation of the p42/p44 MAPK pathway.
**PDEγ-PDEγ Interaction**—One of the structural determinants in PDEγ that may be important for association with c-Src is an SH3-binding site. PDEγ contains an SH3 consensus binding site 20PVTPRKGPP28, which is identical with the corresponding region in the cone isoform with the exception that valine at amino acid position 21 is replaced by threonine. This site may therefore interact with an SH3 domain in c-Src. In previous studies (21) we reported that thrombin also promotes the association of PDEγ with dynamin II. This might also involve interaction of dynamin II SH3-binding site via an intermediate double SH3 domain containing protein (e.g. Grb-2) with 20PVPRKGPP28 in PDEγ. Thus, PDEγ might bind several proteins via SH3 interaction. Indeed, along with c-Src and GRK2, dynamin II is involved in endocytotic processes that are required for certain GPCR/growth factor-dependent activations of p42/p44 MAPK (4–11). However, PDEγ contains only one SH3 domain binding site. It is therefore possible that PDEγ might form either dimers or tetramers, thereby increasing the number of SH3 domain binding sites from 1 to 4. Indeed, it is well established that PDEγ forms dimers via hydrophobic interaction, and indeed, two molecules of PDEγ bind to PDE6 in rod photoreceptors (18). To assess this possibility, we immobilized His-tagged PDEγ on a nickel-agarose matrix in order to capture endogenous PDEγ-c-Src-Grk2 complexes present in lysates from HEK 293 cells. Fig. 4a shows that GRK2 (85 kDa), c-Src (60 kDa), dynamin II (112 kDa), and Grb-2 (26 kDa) in lysates from control cells were all trapped by the His-tagged PDEγ-agarose matrix and eluted with imidazole. The amount of each of these proteins binding to the His-tagged PDEγ-agarose matrix was increased from lysates of cells treated with EGF (fold stimulations in response to EGF versus control cells: GRK2, 1.73 ± 0.51-fold; dynamin II, 1.53 ± 0.18-fold; Grb-2, 1.47 ± 0.14-fold; c-Src, 1.45 ± 0.11-fold, n = 3, P < 0.05 for all versus control). PDEγ was also eluted with imidazole from the nickel matrix (Fig. 4b). The fold increases in the binding of the various proteins are all similar. This is entirely consistent with the possibility that EGF treatment of cells might promote the binding of the entire complex of proteins to PDEγ immobilized on the nickel matrix. Taken together, these findings suggest that EGF may increase the affinity of PDEγ for PDEγ-protein complexes.

We conclude that the stimulation of cells with EGF might induce three events. First, EGF promotes association of GRK2 with the c-Src-PDEγ complex. Second, EGF promotes GRK2-catalyzed phosphorylation of PDEγ, and third, the growth factor may increase the affinity of PDEγ for PDEγ. This may promote formation of a dimeric or tetrameric PDEγ platform upon which other proteins involved in endocytic signaling to p42/p44 MAPK, such as dynamin II, can associate.

**Negative Feedback Regulation by p42/p44 MAPK**—The 20PVTPRKGPP28 site in rod PDEγ also contains a consensus site for phosphorylation by p42/p44 MAPK (20PVTP23). Indeed, we found that this site was phosphorylated by p42/p44 MAPK. Paglia and colleagues (24) have formerly reported stoichiometric phosphorylation of PDEγ by p42 MAPK. Therefore, we have mutated Thr-22 to establish its effect on PDEγ-mediated regulation of p42/p44 MAPK. For this purpose we used cultured airway smooth muscle cells. These cells contain abundant amounts of PDEγ (18), such that the protein is saturating for EGF-stimulated p42/p44 MAPK activation (data not shown). Transfection of these cells with Thr-22 mutant PDEγ-pcDNA3.1 plasmid construct led to a prolonged activation of p42/p44 MAPK by EGF compared with vector (H)-transfected cells (Fig. 5). These data are compatible with the possibility that p42/p44 MAPK can phosphorylate PDEγ at Thr-22 to exert the feedback inhibition of PDEγ-c-Src/GRK2 activity, thereby limiting the duration of p42/p44 MAPK activation in response to EGF. Presumably, mutagenesis of Thr-22 to Ala does not in itself disrupt interaction between PDEγ, c-Src, and GRK2, as this mutant is still capable of supporting activation of p42/p44 MAPK in response to EGF. Indeed, the mutant is more efficient compared with the endogenous wild type PDEγ. In this case, the Thr-22 mutant presumably replaces endogenous wild type PDEγ in the signaling pathway regulating p42/p44 MAPK.

To conclude, these findings highlight an important role for PDEγ in transducing signals from GRK2-c-Src to p42/p44 MAPK. They also demonstrate that PDEγ functions as an important intermediate regulating this mitogenic signaling pathway.

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