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Agonist-Dependent Modulation of G Protein-Coupled Receptor Kinase 2 by Mitogen-Activated Protein Kinases

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ABSTRACT

A variety of G protein-coupled receptors (GPCRs) are phosphorylated by G protein-coupled receptor kinase 2 (GRK2). This event promotes the binding of regulatory proteins termed β-arrestins to GPCRs, leading to uncoupling from G proteins and receptor internalization. Recent data indicate that GRK2 and β-arrestins also play an important role in the stimulation of the extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinase (MAPK) cascade by GPCRs. In this report, we have investigated the existence of functional interactions between GRK2 and MAPK. We show that activation of β_2-adrenergic receptors (β_2-AR) promotes the rapid association of GRK2 and MAPK in living cells, as assessed by coimmunoprecipitation experiments in COS-7 cells transfected with β_2-AR, GRK2, and an epitope-tagged MAPK. Coimmunoprecipitation of MAPK and GRK2 is blocked by inhibition of the MAPK cascade and is not observed upon activation of MAPK in the absence of β_2-AR stimulation, thus indicating that both an active MAPK and agonist occupancy of GPCR are required for the association to occur. Interestingly, we have found that purified ERK1/MAPK can directly phosphorylate the C-terminal domain of GRK2, and that the phosphorylation process is favored by the presence of Gβγ-subunits or an activated receptor. Furthermore, GRK2 phosphorylation by MAPK leads to a decreased activity of GRK2 toward GPCR. Taken together, our results suggest that stimulation of GPCRs promotes the rapid association of GRK2 and MAPK leading to modulation of GRK2 functionality, thus putting forward a new feedback mechanism for the regulation of GPCR signaling.

Activation of G protein-coupled receptors (GPCRs) triggers their interaction with different types of cellular proteins. Interaction with heterotrimeric G proteins promotes its dissociation into Gα and Gβγ subunits, both of which modulate a variety of effector proteins leading to a specific cellular response. On the other hand, GPCR stimulation also induces receptor phosphorylation by a family of specific G protein-coupled receptor kinases (GRKs), followed by binding to the GRK-phosphorylated receptor of cytosolic proteins known as β-arrestins. This promotes receptor uncoupling from heterotrimeric G proteins, a process termed desensitization (Krupnick and Benovic, 1998; Pitcher et al., 1998). GRK2 is a ubiquitous member of the GRK family that has an important role in the modulation of different GPCRs (Aragay et al., 1998; Carman and Benovic, 1998).

Recent data indicate that GRK2 and β-arrestins play additional roles in receptor regulation and signaling. Besides uncoupling from G proteins, the agonist-induced recruitment of GRK2 and β-arrestin to the receptor complex appears to be directly involved in receptor internalization by means of arrestin-mediated targeting of GPCRs to clathrin-coated pits, thus triggering receptor dephosphorylation and recycling to the plasma membrane (Carman and Benovic, 1998; Lefkowitz, 1998; Mayor et al., 1998). On the other hand, emerging evidence suggests that these regulatory proteins are also involved in the process of modulation of mitogen-activated protein kinase (MAPK) cascades by GPCRs. Stimulation of a variety of G_q or G_i-coupled GPCR has been shown to lead to the activation of the extracellular signal-regulated kinases (ERKs) in a Ras-dependent way (Gutkind, 1998; Lefkowitz, 1998; Luttrell et al., 1999a). The recruitment and activation of cytosolic tyrosine kinases of the Src family play a critical role in this process (Gutkind, 1998; Luttrell et al., 1999a). Interestingly, it has been shown recently that β-arrestin can mediate the recruitment of Src to the receptor signaling complex (Luttrell et al., 1999b). This, together with several studies that have indicated that GRK/β-arrestin-mediated receptor internalization is required for the modulation of the ERK/MAPK pathway by various GPCRs (Luttrell et al.,

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ABBREVIATIONS: GPCR, G protein-coupled receptor; β_2-AR, β_2-adrenergic receptor; ERK, extracellular signal-regulated kinase; GRK, GPCR kinase; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PKC, protein kinase C; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmune precipitation buffer; MES, 2-(*N*-morpholino)ethanesulfonic acid.

1997; Daaka et al., 1998; Ahn et al., 1999), suggests an important role for GRKs and arrestins in this signaling pathway.

The activity of the ERK/MAPK pathway is tightly regulated at different levels, including negative MAPK feedback phosphorylation of upstream activators such as Sos1, Raf-1 kinase, and MEK1 by the activated MAPK (Porfiri and McCormick, 1996; Foschi et al., 1997 and references therein). In this regard, GRK2 appears as an important regulatory step in GPCR signaling. GRK2 activity, levels, and subcellular localization are subject to complex regulatory processes, including interaction with G$\beta\gamma$ subunits of G proteins, lipids, agonist-activated receptors, anchoring proteins, and calmodulin (Aragay et al., 1998; Carman and Benovic, 1998; and Pitcher et al., 1998), rapid degradation by the proteasome pathway (Penela et al., 1998) and phosphorylation by other kinases. GRK2 phosphorylation by protein kinase C (PKC) leads to an increased kinase activity toward GPCR, probably due to an enhanced kinase association to the plasma membrane (Chuang et al., 1995; Winstel et al., 1996). On the other hand, agonist stimulation of β_2-adrenergic receptors (β_2-AR) triggers the rapid tyrosine phosphorylation of GRK2 by Src, which results in an enhancement of GRK2 intrinsic activity (Sarnago et al., 1999).

In this context, we have explored the existence of additional mechanisms that would modulate GPCR stimulation of the ERK/MAPK pathway at the GRK2 level. We report that activation of β_2-AR promotes the presence of active MAPK and GRK2 in the same multimolecular complex. We also show that MAPK phosphorylates GRK2 in vitro in its C-terminal domain, and that MAPK-phosphorylated GRK2 displays a reduced activity toward activated GPCRs. In addition to recent results describing the regulation of β-arrestin-1 function by ERKs (Lin et al., 1999), our results put forward a new autoregulatory loop in the GPCR-MAPK cascade signaling pathway.

Experimental Procedures

Materials. Bovine GRK2 was overexpressed and purified from baculovirus-infected Sf9 cells as described (Murga et al., 1996). Purity of the GRK2 preparation as determined by SDS-polyacrylamide gel electrophoresis (PAGE) was >95%. Recombinant baculovirus for GRK2 and purified G$\beta\gamma$ subunits from bovine brain were kindly provided by Dr. J. L. Benovic at the Thomas Jefferson Cancer Institute of Philadelphia. GST fusion proteins containing amino acids 50–145 (FP1) and 437–689 (FP2) of GRK2 were generated and purified as reported (Murga et al., 1996). The cDNAs encoding human hemagglutinin (HA)-MAPK (ERK1) and the constitutively active double MEK1 mutant S218/222D (Catling et al., 1995) were provided by Dr. J. Moscat (Centro de Biología Molecular, Madrid, Spain). Wild-type MEK and a dominant-negative MEK mutant (Cowley et al. 1994) were provided by Dr. J. M. Redondo (Centro de Biología Molecular, Madrid, Spain). COS-7 cells were from the American Type Culture Collection (Manassas, VA). Culture media and LipofectAMINE were from Life Technologies, Inc. (Gaithersburg, MD). Protein A-Sepharose, isoproterenol, and heparin (mol. wt. 6,000) were obtained from Sigma (St. Louis, MO). [γ-^{32}P]ATP was purchased from Amersham Corp. (Buckinghamshire, England). Purified activated MAPK was obtained from Stratagene Laboratories (La Jolla, CA), and the MEK inhibitors PD98059 and U0126 were purchased from Calbiochem (La Jolla, CA) and Promega (Madison, WI), respectively. All other reagents were of the highest grade commercially available.

Cell Culture and Transfection. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified 7% CO_2 atmosphere. Transfections were performed on 70% confluent monolayers as reported (Sarnago et al., 1999). Transient expression was confirmed by immunoblot analysis of whole-cell lysates using specific antisera [polyclonal HA-probe Y11 for MAPK from Santa Cruz Laboratories (Santa Cruz, CA) and Ab9 (Ruiz-Gómez and Mayor, 1997) for GRK2].

Cell Treatments. Isoproterenol stimulation of COS-7 cells was performed 48 h after transfection at 37°C in culture medium supplemented with 20 mM HEPES (pH 7.5) and 1 mM ascorbic acid (Sigma). Epidermal growth factor (100 ng/ml, from Upstate Biotechnology) was added to cells after overnight serum-starving. Treatments with the MEK inhibitor PD98059 (50 μM) were performed at 37°C during 45 min before isoproterenol stimulation and those with the U0126 inhibitor as reported (Favata et al., 1998).

Immunoprecipitation and Western Blotting. For immunoprecipitation, the cells were washed twice with ice-cold phosphate-buffered saline supplemented with 1 mM sodium orthovanadate, solubilized in 700 μl/100-mm dish of RIPA buffer (200 mM MES, pH 6.2, 1% (v/v) Triton X-100, 0.1 mM $MgCl_2$, 0.3 mM NaCl, 0.1 mM EGTA, 0.5% deoxycholate, 10 mM NaF, 1 mM Na_3VO_4, and a cocktail of protease inhibitors). After gentle rocking for 90 min at 4°C, the lysates were clarified by centrifugation, and an aliquot (30 μl) was used to assess protein overexpression. Tagged MAPK was immunoprecipitated from radioimmune precipitation (RIPA) buffer lysates by overnight incubation with a specific anti-HA antibody (clone 12CA5) in the presence of 0.5 mg/ml bovine serum albumin. After incubation at 4°C with protein A-Sepharose for 1 h and centrifugation, the beads were washed with ice-cold RIPA buffer and resuspended in SDS sample buffer. All immunoprecipitated samples were boiled for 5 min before resolution by 10% SDS-PAGE and transference to nitrocellulose membranes. The presence of HA-MAPK and GRK2 in the immunoprecipitates was analyzed by using the anti-HA and Ab9 antibodies, respectively. Blots were developed using a chemiluminescent method (ECL; Amersham).

Determination of MAPK Activity and in Vitro Phosphorylation Experiments. For detection of MAPK activity in control and stimulated samples, anti-HA immunoprecipitates (40-μl aliquots) were washed with phosphorylation buffer to remove the detergent, and the MAPK-specific substrate myelin basic protein (MBP-1) was added (40 μg). The phosphorylation reaction was initiated by adding 40 μl of kinase reaction buffer to a final concentration of 40 mM MES, pH 6.2, 10 mM magnesium acetate, 2.5 mM EGTA, 5 mM NaF, 50 μM ATP, and 5,000 cpm/pmol [γ-^{32}P]ATP. After incubation for 30 min at 30°C, the reaction was stopped with 2× SDS-sample buffer, and the phosphorylated proteins were resolved by SDS-PAGE, revealed by autoradiography, and quantitated by densitometry. For the in vitro phosphorylation studies, recombinant GRK2 protein at the concentrations indicated in the figure legends was incubated with purified MAPK at a final concentration of 2.5 ng/μl (56.8 nM) in a final volume of 40 μl of kinase reaction buffer (25 mM HEPES, pH 7.2, 10 mM magnesium acetate, and 50 μM ATP) in the presence or absence of 0.25 ng/μl heparin to inhibit GRK2 autophosphorylation (Sarnago et al., 1999). The reaction was initiated by adding 2 μl of a 1/10 dilution of [γ-^{32}P]ATP (10 μCi/μl; Amersham). After 30 min at 30°C, the reaction was stopped by the addition of 2× SDS-PAGE sample buffer. For the phosphorylation of the GST-GRK2 fusion proteins FP1 and FP2 or GST (control), these purified proteins were added to the reaction buffer in the same conditions and at a final concentration of 2 μM in the absence or presence of 100 nM G$\beta\gamma$ subunits purified from bovine brain. Phosphorylated proteins were resolved by electrophoresis in 8% polyacrylamide gels and visualized by autoradiography. The activity of control or MAPK-phosphorylated GRK2 aliquots was assessed by performing a rhodopsin phosphorylation assay as previously described (Murga et al., 1996).

Results and Discussion

The activation of β_2-AR leads to its rapid phosphorylation by GRK2 and also to stimulation of the ERK/MAPK pathway (Daaka et al., 1998; Luttrell et al., 1999b). To explore potential functional interactions between GRK2 and MAPK, we investigated whether these two proteins would coimmunoprecipitate upon receptor activation. COS-7 cells were transiently transfected with β_2-AR, GRK2 and epitope-tagged HA-MAPK (ERK1) and treated with the β-agonist isoproterenol for different periods of time. HA-MAPK was then immunoprecipitated with specific anti-HA antibodies and the presence of GRK2 assessed by immunoblot analysis. Figure 1 shows that isoproterenol stimulation promotes the rapid association of GRK2 and HA-MAPK, which is maximal (≈3-fold over basal values) within 5 min of agonist exposure. A certain level of GRK2 and MAPK association is also detected under control conditions, likely due to the basal activity of the overexpressed β_2-ARs (Ruiz-Gómez and Mayor, 1997). GRK2 was not detected in the immunoprecipitates when a nonrelated monoclonal antibody was used instead of anti-HA antibodies (data not shown). The rapid kinetics of GRK2/MAPK coimmunoprecipitation is consistent with the time course of β_2-AR-mediated activation of HA-MAPK activity in our experimental conditions, as assessed by testing the activity of HA-MAPK immunoprecipitates toward myelin basic protein (data not shown), in agreement with previously reported data (Daaka et al., 1998; Ahn et al., 1999; Luttrell et al., 1999b). The observed effect is also in the same time range of other related cellular signals promoted by GPCR activation, such

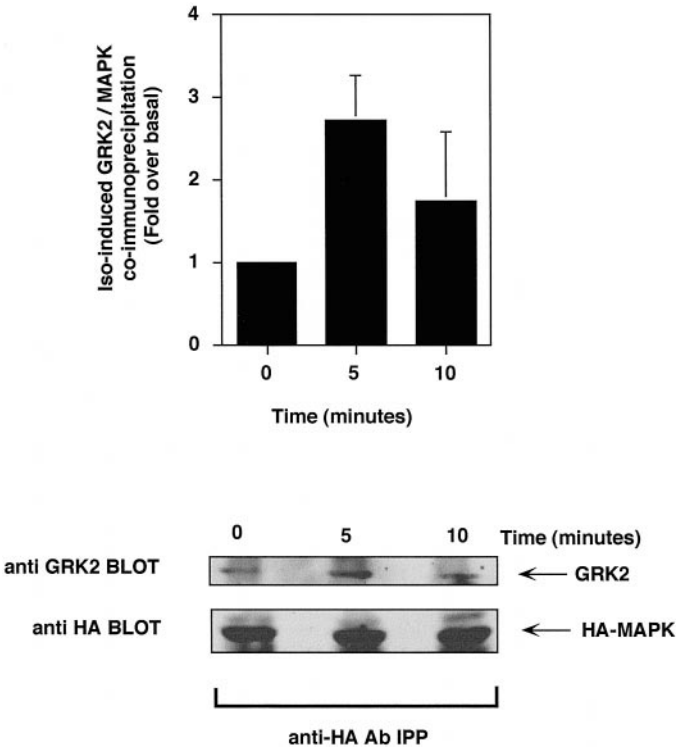

Fig. 1. Agonist-induced coimmunoprecipitation of GRK2 and MAPK. COS-7 cells transiently transfected with GRK2, HA-MAPK, and β_2-AR (1:2:2 ratio) were incubated for the indicated times in the absence or presence of 10 μM isoproterenol as detailed under *Experimental Procedures*. Tagged MAPK immunoprecipitates from RIPA buffer lysates were resolved by 10% SDS-PAGE, blotted, and analyzed with specific anti-GRK2 antibodies (top panel). The amount of HA-MAPK present in each sample was determined by immunoblotting using a monoclonal anti-HA antibody (bottom panel). The amount of coimmunoprecipitated GRK2 was measured by scanner laser densitometry, and the data normalized to the amount of HA-MAPK protein present in the immunoprecipitates. Nonstimulated controls were taken as the basal condition. Each data represent the mean ± S.E. from three to six independent experiments. A representative experiment is shown in the gel, with the arrows indicating the migration of GRK2 and HA-MAPK.

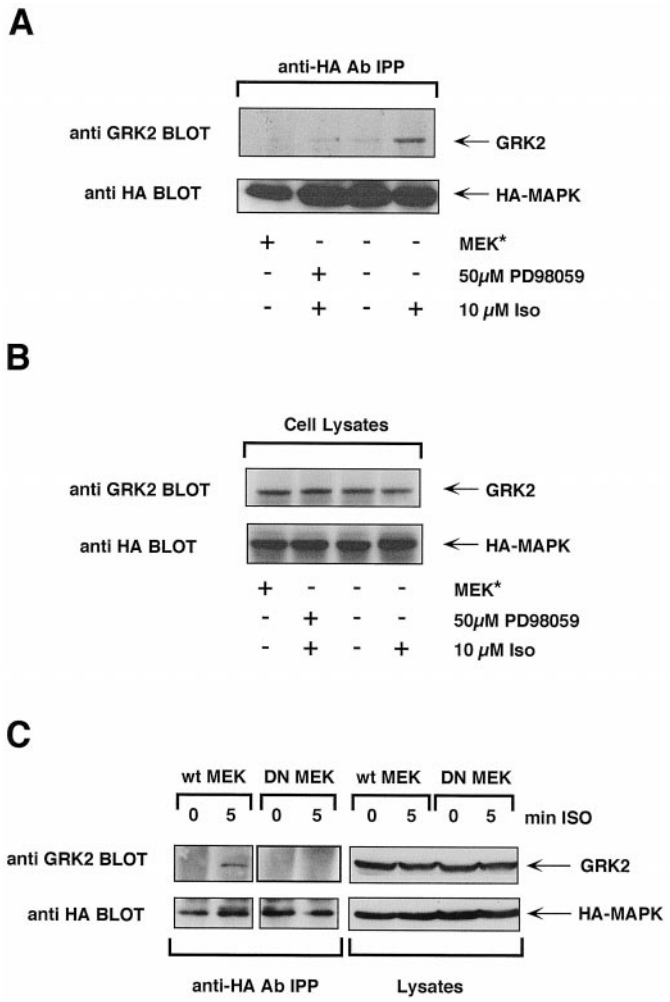

Fig. 2. GRK2 and MAPK association requires an active MAPK and receptor stimulation. A, COS-7 cells were transiently transfected with different combinations of GRK2, active MEK1 (MEK*), HA-MAPK, and β_2-AR as indicated, and stimulated or not with isoproterenol (10 μM) for 5 min in the absence or presence of the MEK inhibitor PD098059 (50 μM) as detailed under *Experimental Procedures*. HA-MAPK was immunoprecipitated from RIPA buffer lysates, and samples were resolved by 10% SDS-PAGE, blotted, and analyzed with anti-GRK2 antibodies (top panel). The presence of HA-MAPK was analyzed in the same gel using a specific anti-HA antibody (bottom panel). The arrows indicate the migration of GRK2 and HA-MAPK. A gel representative of three independent experiments is shown. B, aliquots of cell lysates from the experiment shown in panel A were resolved by SDS-PAGE, blotted, and analyzed for GRK2 and HA-MAPK expression levels as detailed in *Methods*. The arrows indicate the migration of GRK2 and HA-MAPK. C, COS-7 cells were transiently transfected with GRK2, HA-MAPK, β_2-AR and wild-type (wt) MEK or dominant-negative (DN) MEK as indicated, and stimulated or not with isoproterenol (10 μM) for 5 min. HA-MAPK immunoprecipitates and aliquots of the RIPA lysates were obtained and resolved as above, blotted, and analyzed with anti-GRK2 antibodies as indicated. The presence of HA-MAPK was analyzed in the same gel using a specific anti-HA antibody (bottom panel). The arrows indicate the migration of GRK2 and HA-MAPK. A gel representative of three independent experiments is shown.

as Src-dependent phosphorylation of Shc, Gab1, dynamin, or GRK2 (see Sarnago et al., 1999 and references therein).

As a first step to understanding the mechanisms leading to the association of GRK2 and MAPK in β_2-AR-stimulated cells, we tested the effects of some modulators of the ERK/MAPK cascade on GRK2/MAPK coimmunoprecipitation. Interestingly, the marked GRK2/MAPK association promoted by isoproterenol is strongly inhibited in the presence of the MEK inhibitors PD98059 (Fig. 2A) or U0126 (data not shown). Furthermore, cotransfection of dominant-negative MEK strongly inhibits the agonist-induced GRK2/MAPK coimmunoprecipitation (Fig. 2C). Taken together, our data indicate that an activated MAPK is necessary for the association to occur. However, MAPK activation per se is not sufficient to promote HA-MAPK/GRK2 coimmunoprecipitation, because cotransfection of a constitutively active MEK mutant did not trigger the association of MAPK and GRK2 in the absence of β_2-AR and isoproterenol stimulation (Fig. 2A). In this regard, cell stimulation with epidermal growth factor in the absence of β_2-AR activation leads to increased MAPK activity (as assessed by myelin basic protein phosphorylation) but not GRK2/MAPK association (data not shown), furthermore indicating that GPCR activation is required for the interaction of these kinases. The observed effects are not due to changes in HA-MAPK immunoprecipitation or in the expression levels of GRK2 and MAPK under these experimental conditions (Fig. 2, B and C). The requirement of both GPCR stimulation and an active MAPK to observe its interaction with GRK2 indicates that these events promote relevant changes in the subcellular localization, conformation, or association to other cellular proteins of GRK2 and/or MAPK, that allow the presence of these kinases in the same multimolecular complex. More experiments would be needed to delimitate the localization and composition of the GRK2/MAPK complex.

Which are the functional consequences of the association between of GRK2 and MAPK? To address this question, we incubated recombinant purified preparations of GRK2 and MAPK (ERK1) under phosphorylating conditions. Figure 3A shows that the presence of MAPK promotes a clear increase in GRK2 phosphorylation, which is likely due to the activity of MAPK, because the experiments were performed in the

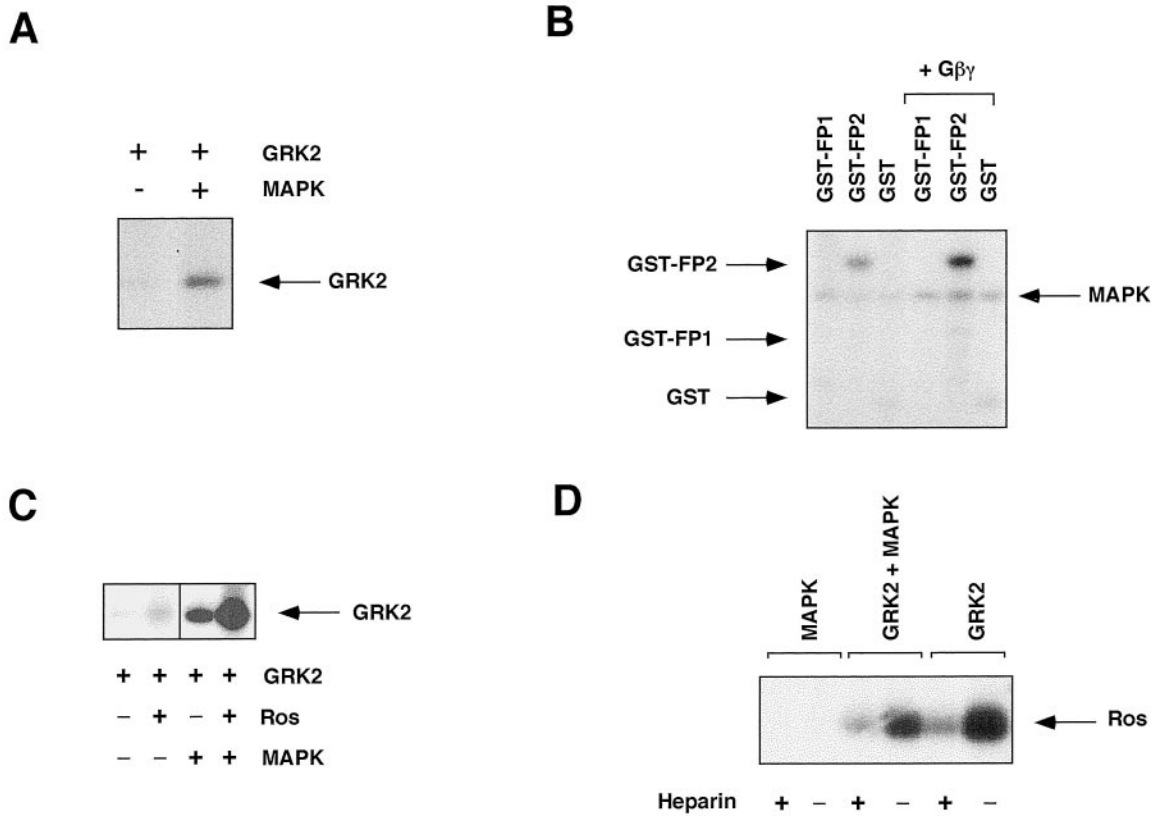

Fig. 3. In vitro phosphorylation of GRK2 by MAPK and functional consequences. A, increased GRK2 phosphorylation in the presence of MAPK. Recombinant GRK2 (60 nM) was incubated with purified MAPK (56 nM) as detailed in *Methods* for 30 min at 30°C in 40 μl of phosphorylation buffer in the presence of 0.25 ng/μl heparin to inhibit GRK2 autophosphorylation, followed by SDS-PAGE and autoradiography. B, MAPK phosphorylates the C-terminal region of GRK2. GST-GRK2 fusion proteins encompassing GRK2 residues 50–147 (FP1) and 437–689 (FP2) or GST (control) were incubated at a final concentration of 2 μM with 56 nM purified MAPK in phosphorylation buffer as described above in the absence or presence of purified G$\beta\gamma$ subunits (100 nM). Phosphorylated proteins were resolved by electrophoresis in 8% polyacrylamide gels and visualized by autoradiography. The arrows indicate the migration of the fusion proteins and MAPK. A representative gel of at least two independent experiments is shown. C, the phosphorylation of GRK2 by MAPK is increased in the presence of bleached rhodopsin. GRK2 (60 nM) was incubated under phosphorylation conditions in the presence or absence of MAPK (56 nM) and/or light-activated rod-outer segments (Ros) as indicated. Proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography. The gel is representative of two independent experiments. D, decreased GRK2 activity upon MAPK phosphorylation. GRK2 (60 nM) was preincubated for 30 min at 30°C in phosphorylation buffer lacking radioactive ATP in the presence or absence of MAPK. Aliquots were diluted 1:5 in GRK2 phosphorylation buffer and tested for activity toward purified, light-activated rhodopsin (0.5 μM) in the presence or absence of heparin as indicated. After 20 min at 30°C, reactions were stopped by addition of SDS-PAGE sample buffer and phosphorylated rhodopsin detected by electrophoresis on 12% polyacrylamide gels followed by autoradiography. The gel is representative of two independent experiments.

presence of heparin, an inhibitor of GRK2 activity and autophosphorylation. The stoichiometry attained was in the range of 0.2 to 0.6 mol of Pi/mol of GRK2, depending on the GRK2 preparation. Control experiments indicated that heparin had no effect on MAPK activity toward other substrates such as PHAS-I (data not shown). Analysis of the GRK2 sequence revealed the existence of several potential consensus phosphorylation sites for MAPK in the C-terminal domain region, including an optimal consensus site [PX(S/T)P] at Ser-670. Consistently, a GST fusion construct encompassing the C-terminal region of GRK2 (FP2) was readily phosphorylated by purified MAPK (Fig. 3B), whereas this kinase was unable to phosphorylate GST or a GST fusion protein comprising an N-terminal region of GRK2 (FP1). The C-terminal region of GRK2 has been shown to be directly involved in the interaction of this kinase with G$\beta\gamma$ subunits, which appear to play a key role in GRK2 activation and translocation to the plasma membrane upon GPCR stimulation (Koch et al., 1993; Daaka et al., 1997). Interestingly, the presence of purified G$\beta\gamma$ subunits promoted a marked (2–3-fold) increase in the phosphorylation of GST-FP2 by MAPK, although showing no effect on GST-FP1 or GST phosphorylation (Fig. 3B). Because GRK2 activation involves its interaction with both agonist-occupied GPCR and G$\beta\gamma$-subunits, we compared GRK2 phosphorylation by MAPK in the absence or presence of light-activated rhodopsin. An increased GRK2 phosphorylation is observed in the presence of activated rhodopsin, which is not due to an enhanced kinase autophosphorylation in these experimental conditions (see control in Fig. 3C). Together with the data in living cells indicating that β_2-AR activation is required to promote GRK2/MAPK coimmunoprecipitation, these results suggest that agonist occupancy of GPCR would favor the interaction of these kinases and the subsequent GRK2 phosphorylation by MAPK.

We next explored whether GRK2 phosphorylation by MAPK had any effect on GRK2 activity toward rhodopsin, as a model for activated GPCR. As shown in Fig. 3D, the presence of purified MAPK, which does not phosphorylate rhodopsin, promoted a significant decrease (~40% compared with GRK2 alone) in the phosphorylation of light-activated rhodopsin by GRK2. Rhodopsin phosphorylation under the different experimental conditions is only due to the activity of GRK2, because it is blocked in the presence of the GRK2 inhibitor heparin (Fig. 3D). Overall, these data suggest that MAPK phosphorylation of GRK2 would lead to a decreased activity of the latter toward activated GPCR, and/or to a reduced ability of MAPK-associated GRK2 to interact with stimulated receptors. Nevertheless, we cannot rule out the possibility that such phosphorylation events would also affect other GRK2 cellular functions.

Taken together, our data in vitro and in living cells suggest that activation of GPCRs would promote the presence of active MAPK and GRK2 in the same multimolecular complex, this leading to GRK2 phosphorylation by MAPK and changes in GRK2 functionality. In this regard, it is worth noting that during the revision process of this manuscript, Pitcher et al. (1999) have reported that a fraction of the GRK2 cellular pool is phosphorylated at a MAPK consensus phosphorylation site (Ser-670) in Sf9 cells and that phosphorylation of GRK2 by MAPK impairs the ability of GRK2 to phosphorylate soluble and membrane-bound substrates and attenuates G$\beta\gamma$-mediated activation of GRK2. Our results are consistent with these data and, in addition, demonstrate that activation of G protein-coupled receptors promotes the rapid association of GRK2 and active MAPK in living cells. The existence of such an autoregulatory loop in the GPCR/MAPK pathway is also consistent with a recent report by Lefkowitz and coworkers (Lin et al., 1999) showing an inhibitory feedback regulation of β-arrestin-1 function by ERK/MAPK. A decreased activity of GRK2 and β-arrestin on MAPK phosphorylation would attenuate the coupling of GPCR to the ERK/MAPK pathway by decreasing Src recruitment and/or receptor internalization (Luttrell et al., 1999a,b).

Furthermore, our results suggest that GPCR activation by agonists leads to the formation of multimolecular receptor signaling complexes with different compositions, which can stimulate different intracellular signaling pathways and also promote different autoregulatory mechanisms. At the level of GRK2, we have recently shown that activation of β_2-AR promotes its rapid phosphorylation by *c*-Src on tyrosine residues, which results in an enhancement of GRK2 intrinsic activity (Sarnago et al., 1999). It is tempting to speculate that such a mechanism would provide a positive feedback loop for the modulation of the MAPK cascade by GPCR, by reinforcing β-arrestin binding and Src recruitment, and contributing to signal switching from G proteins to the MAPK pathway (Lefkowitz, 1998). In this context, this report suggests that rapid agonist-induced association of GRK2 and MAPK would exert an inhibitory control of GRK2 function. Overall, these data underscore a stringent control of GRK2 functionality triggered by GPCR activation, consistent with an important physiological role for this kinase. In this regard, the functional interactions between Src or MAPK and GRK2 may be relevant to better understand the physiological consequences of the disruption of the GRK2 gene in mice (an embryonic lethal phenotype due to myocardial hypoplasia) (Jaber et al., 1996) or of the increased levels of this kinase detected in congestive heart failure patients and in experimental models of cardiac hypertrophy (Ungerer et al., 1993; Choi et al., 1997; Rockman et al., 1998). A better knowledge about how the different mechanisms of GRK2 regulation inherent to GPCR stimulation are combined and integrated at the cellular level could shed new light in our understanding of GPCR modulation and signaling.

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References

Ahn S, Maudsley S, Luttrell LM, Lefkowitz RJ and Daaka Y (1999) Src-mediated tyrosine phosphorylation of dynamin is required for β2-adrenergic receptor internalization and mitogen-activated protein kinase signaling. *J Biol Chem* **274:**1185–1188.

Aragay AM, Ruiz-Gómez A, Penela P, Sarnago S, Elorza A, Jiménez-Sainz MC and Mayor F Jr (1998) G protein-coupled receptor kinase 2 (GRK2): Mechanisms of regulation and physiological functions. *FEBS Lett* **430:**37–40

Carman CV and Benovic JL (1998) G-protein-coupled receptors: Turn-ons and turn-offs. *Curr Opin Cell Biol* **8:**335–344.

Catling AD, Schaeffer HJ, Reuter CWM, Reddy GR and Weber MJ (1995) A proline-rich sequence unique to MEK1 and MEK2 is required for raf binding and regulates MEK function. *Mol Cell Biol* **15:**5214–5225.

Choi D-J, Walter JK, Hunter JJ and Rockman HA (1997) Mechanism of β-adrenergic receptor desensitization in cardiac hypertrophy is increased β-adrenergic receptor kinase. *J Biol Chem* **272:**17223–17229.
Chuang TT, LeVine H 3rd and De Blasi A (1995) Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C. *J Biol Chem* **270:**18660–18665.
Cowley S, Paterson H, Kemp P and Marshall CJ (1994) Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77:**841–852.
Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SSG, Caron MG and Lefkowitz RJ (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* **273:**685–688
Daaka Y, Pitcher JA, Richardson M, Stoffel RH, Robishaw JD and Lefkowitz RJ (1997) Receptor and G betagamma isoform-specific interactions with G protein-coupled receptor kinases. *Proc Natl Acad Sci USA* **94:**2180–2185.
Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyck DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA and Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* **273:**18623–18632.
Foschi M, Chari S, Dunn MJ and Sorokin A (1997) Biphasic activation of p21ras by endothelin-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase. *EMBO J* **21:**6439–6451.
Gutkind JS (1998) The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* **273:**1839–1842.
Jaber M, Koch WJ, Rockman H, Smith B, Bond RA, Sulik KK, Ross J jr, Lefkowitz RJ, Caron MG and Giros B (1996) Essential role of β-adrenergic receptor kinase 1 in cardiac development and function. *Proc Natl Acad Sci USA* **93:**12974–12979.
Koch WJ, Inglese J, Stone WC and Lefkowitz RJ (1993) The binding site for the beta gamma subunits of heterotrimeric G proteins on the beta-adrenergic receptor kinase. *J Biol Chem* **268:**8256–8260.
Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:**289–319.
Lefkowitz RJ (1998) G protein-coupled receptors. *J Biol Chem* **273:**18677–18680.
Lin F-T, Miller WE, Luttrell LM and Lefkowitz J (1999) Feedback regulation of β-arrestin1 function by extracellular signal-regulated kinases. *J Biol Chem* **274:**15971–15974.
Luttrell LM, Daaka Y, Della Rocca GJ and Lefkowitz RJ (1997) G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. *J Biol Chem* **272:**31648–31656.
Luttrell LM, Daaka Y and Lefkowitz RJ (1999a) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* **11:**177–183.
Luttrell LM, Ferguson SSG, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F-T, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ (1999b) β-arrestin-dependent formation of 2Adrenergic receptor-src protein kinase complexes. *Science* **283:**655–661.
Mayor F Jr, Penela P and Ruiz-Gómez A (1998) Role of G protein-coupled receptor kinase 2 and arrestins in β-adrenergic receptor internalization. *Trends Cardiovasc Med* **8:**234–240.
Murga C, Ruiz-Gómez A, García-Higuera I, Kim CM, Benovic JL and Mayor F Jr (1996) High affinity finding of β-adrenergic receptor kinase to microsomal membranes. *J Biol Chem* **271:**985–994.
Penela P, Ruiz-Gómez A, Castaño JG and Mayor F Jr (1998) Degradation of the G protein-coupled receptor kinase 2 by the proteasome pathway. *J Biol Chem* **273:**35238–35244
Pitcher JA, Freedman NJ and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Annu Rev Biochem* **67:**653–692.
Pitcher JA, Tesmer JJG, Freeman JLR, Capel WD, Stone WC and Lefkowitz RJ (1999) Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases. *J Biol Chem* **274:**34531–34534.
Porfiri E and McCormick F (1996) Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor hSOS1. *J Biol Chem* **271:**5871–5877.
Rockman HA, Chien KR, Choi DJ, Iaccarino G, Hunter JJ, Ross J Jr, Lefkowitz RJ and Koch WJ (1998) Expression of a β-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. *Proc Natl Acad Sci USA* **12:**7000–7005.
Ruiz-Gómez A and Mayor F Jr (1997) β-adrenergic receptor kinase (GRK2) colocalizes with β-adrenergic receptors during agonist-induced receptor internalization. *J Biol Chem* **272:**9601–9604.
Sarnago S, Elorza A and Mayor F Jr (1999) Agonist-dependent phosphorylation of the G protein-coupled receptor kinase 2 (GRK2) by src tyrosine kinase. *J Biol Chem* **274:**34411–34416.
Ungerer M, Bohm M, Elce JS, Erdmann E and Lohse MJ (1993) Alttered expression of β-adrenergic receptor kinase and β1-adrenergic receptors in the failing human heart. *Circulation* **87:**454–463.
Winstel R, Freund S, Krasel C, Hoppe E and Lohse MJ (1996) Protein kinase cross-talk: Membrane targeting of the β-adrenergic receptor kinase by protein kinase C. *Proc Natl Acad Sci USA* **93:**2105–2109.

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