Feedback Inhibition of G Protein-coupled Receptor Kinase 2 (GRK2) Activity by Extracellular Signal-regulated Kinases*

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G protein-coupled receptor kinase (GRK)-mediated receptor phosphorylation and β-arrestin binding uncouple G protein-coupled receptors (GPCRs) from their respective G proteins and initiate the process of receptor internalization. In the case of the β2-adrenergic receptor and lysophosphatidic acid receptor, these processes can lead to ERK activation. Here we identify a novel mechanism whereby the activity of GRK2 is regulated by feedback inhibition. GRK2 is demonstrated to be a phosphoprotein in cells. Mass spectrometry and mutational analysis localize the site of phosphorylation on GRK2 to a carboxyl-terminal serine residue (Ser670). Phosphorylation at Ser670 impairs the ability of GRK2 to phosphorylate both soluble and membrane-incorporated receptor substrates and dramatically attenuates Gβγ-mediated activation of this enzyme. Ser670 is located in a peptide sequence that conforms to an ERK consensus phosphorylation sequence, and in vitro, in the presence of heparin, ERK1 phosphorylates GRK2. Inhibition of ERK activity in HEK293 cells potentiates GRK2 activity, whereas, conversely, ERK activation inhibits GRK2 activity. The discovery that ERK phosphorylates and inactivates GRK2 suggests that ERK participates in a feedback regulatory loop. By negatively regulating GRK-mediated receptor phosphorylation, β-arrestin-mediated processes such as Src recruitment and clathrin-mediated internalization, which are required for GPCR-mediated ERK activation, are inhibited, thus dampening further ERK activation.

G protein-coupled receptor kinases (GRKs) constitute a family of six mammalian serine/threonine protein kinases that phosphorylate agonist-occupied G protein-coupled receptors (reviewed in Ref. 1). GRK-phosphorylated GPCRs bind stoichiometrically to inhibitory proteins known as arrestins. The binding of β-arrestin1 or -2, extraretinal isoforms, prevents receptor-mediated G protein activation, recruits other molecules such as Src to the receptor, and serves to target the phosphorylated receptor for internalization via clathrin-coated pits (2). The recruitment of Src and engagement of the clathrin-coated pit system by the phosphorylated β-adrenergic receptor (β2AR) have been shown to be essential for β2AR-mediated ERK activation (3). The GRKs thus play a critical role in regulating GPCR-mediated signal transduction. Following agonist exposure, GRK-mediated receptor phosphorylation leads to an attenuation of G protein-mediated signaling (receptor desensitization) while at the same time initiating receptor-mediated ERK activation.

The activity of the GRKs is tightly regulated via a number of mechanisms. These include regulation by the βγ subunits of heterotrimeric G proteins (Gβγ), lipids (phosphatidylinositol 4,5-bisphosphate and phosphatidyserine), and cytoskeletal (actin) and calcium-binding proteins (recoverin, calmodulin), as well as by protein kinase C (reviewed in Ref. 1). In most cases regulation of the GRKs is subfamily-specific. Here we identify a previously unappreciated and apparently specific mechanism by which GRK2 activity is regulated. GRK2 is demonstrated to exist as a phosphoprotein in cells. Mapping the site of phosphorylation, elucidating the functional consequences of this phosphorylation event, and identifying the GRK2 kinase in cells yields new insight into the regulatory mechanisms controlling GPCR-mediated signal transduction.

EXPERIMENTAL PROCEDURES

Materials—GRK2 was purified from baculovirus-infected SF9 cells as described (4). Rod outer segment (ROS) membranes (5), Gβγ (6), tubulin (7), and β2AR (8, 9) were purified as described previously. ERK1 was a generous gift of Melanie H. Cobb (University of Texas Southwestern Medical Center), and the dominant-negative ERK/MAP kinase 1 (MEK1(MEK1(K97A))) and constitutively active MEK1 (MEK1(S218D/S222D)) constructs were provided by Edwin G. Krebs (University of Washington) and Raymond L. Erikson (Harvard University), respectively.

Construction of Wild Type and Mutant GRK2 cDNAs—Using polyclonal chain reaction, a Kozak sequence was added to bovine GRK2 and inserted into the EcoRI BamHI fragment of the respective pRK5 construct into pVL1392. ERK1-mediated phosphorylation of GRK2—Purified phosphorylated or unphosphorylated GRK2 (2 μM) was incubated with purified ERK1 (30 μM) in 40 mM HEPES, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol containing 60 μM [γ-32P]ATP (~1000 cpm/mmol) in a final volume of 25 μl. Incubations were performed at 37 °C for 45 min. The stoichiometry of ERK-mediated GRK2 phosphorylation was determined by PhosphorImager analysis (Molecular Dynamics). When the

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1 The abbreviations used are: GRK, G protein-coupled receptor kinase; GPCR, G protein-coupled receptor; β2AR, β2-adrenergic receptor; Gβγ, the βγ subunits of heterotrimeric G proteins; ERK, extracellular signal-regulated kinase; MEK, ERK kinase kinase; ROS, rod outer segment.
activity of ERK-phosphorylated GRK2 was assessed, radiolabeled ATP was omitted and reactions were terminated by desalting on d-Salt Polyacrylamide 6000 Plastic Desalting columns (Pierce) to remove heparin and ATP. GRK2 remaining in solution following the desalting procedure was determined by quantitative Western blot using anti-GRK2 antibodies (10). A 32P-labeled phosphorylation reaction was performed alongside nonradioactive reactions to assess the stoichiometry of ERK-mediated GRK2 phosphorylation.

**RESULTS AND DISCUSSION**

**GRK2 Is a Phosphoprotein in Cells**—GRK2 was purified from baculovirus-infected Sf9 cells using previously published procedures (4). The recombinant protein appeared pure by Coomassie Blue staining, but isoelectric focusing revealed two distinct major bands and a number of minor associated bands. GRK2 purified enzyme was resolved into two peaks of GRK2 protein and activity on Source-S (Fig. 1). Notably, as compared with the first peak (P1), the second peak (P2) exhibited a higher affinity for substrate than P2 as reflected in the lower $K_m$ of phosphorylated GRK2 for $\beta_2$AR and tubulin. The maximal rate of substrate phosphorylation ($V_{max}$) was, however, significantly lower for P1 than P2. The most profound difference was observed when $\beta_2$AR, in the presence of G$\beta\gamma$ subunits, was utilized as substrate. Under these conditions P1 had an approximately 8-fold lower $V_{max}$ than P2 (Table I).

We next examined the dose dependent activation of the two GRK2 species by G$\beta\gamma$. As shown in Fig. 2A, P1 exhibits dramatically impaired G$\beta\gamma$-mediated activation of GRK2. These results reveal a profound functional consequence of GRK2 phosphorylation, an impairment of the rate of GRK2-mediated phosphorylation, and further suggest that this modification may serve to attenuate receptor-mediated allosteric activation of GRK2.

**Identification of Ser$^{670}$ as the Site Phosphorylated on GRK2**—To demonstrate that Ser$^{670}$ represents a site of phosphorylation, two mutant forms of GRK2 were constructed. In these mutants one of the candidate sites of phosphorylation, Ser$^{676}$, was mutated to either an alanine (GRK2(S670A)) or an aspartic acid residue (GRK2(S670D)). If Ser$^{670}$ is a site of phosphorylation, the mutation of Ser$^{676}$ would be predicted not to affect the phosphorylation status of GRK2. Conversely, mutating this site to an aspartic acid residue would be predicted to produce an enzyme that mimics phosphorylated GRK2. If Ser$^{670}$, and not Ser$^{676}$, is a site of phosphorylation, then mutation of Ser$^{670}$ would be predicted not to affect the phosphorylation status of GRK2. The two mutant forms of GRK2 were expressed in and purified from Sf9 cells. The chromatographic profile of these enzymes on Source-S and their responsiveness to G$\beta\gamma$ subunits when rhodopsin was used as a substrate was subsequently examined. GRK2(S670A) and GRK2(S670D) chromatographed as single peaks on Source-S eluting at 100 mM NaCl for GRK2(S670A) and 70 mM NaCl for GRK2(S670D). These elution positions are identical to P2 and P1, respectively. Observations consistent with the identification of Ser$^{670}$ as the site of GRK2 phosphorylation. Furthermore, the G$\beta\gamma$ sensitivity of GRK2(S670A) and GRK2(S670D) is es-

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**TABLE I**

| Substrate |
|-----------|
| **Phosphorylated GRK2 (P1)** | **Unphosphorylated GRK2 (P2)** |
| $K_m$ (mM) | $V_{max}$ (pmol/min/mg) |
| $\beta_2$AR | $0.05 \pm 0.01$ | $0.02 \pm 0.01$ |
| $\beta_2$AR + G$\beta\gamma$ | $0.04 \pm 0.02$ | $0.05 \pm 0.03$ |
| Tubulin | $0.04 \pm 0.03$ | $0.15 \pm 0.03$ |

**FIG. 1.** Chromatography on Source-S resolves two forms of GRK2. Purified GRK2 (~15 mg) was loaded on a Source-S column equilibrated in 20 mM HEPES, pH 7.5, 5 mM EDTA, 20 mM NaCl + protease inhibitors, the column was developed using a 20–150 mM NaCl gradient (125 ml), and 1.0 ml fractions were collected. The protein content and GRK2 activity of the eluted fractions are shown. The inset shows the elution positions of GRK2 as determined by Coomassie Blue staining.
sententially identical to that observed using the unphosphorylated (P2) and phosphorylated (P1) forms of wild type GRK2 (compare Fig. 2, panels A and B). These results identify Ser670, a potential site of ERK phosphorylation, as the GRK2 residue phosphorylated in Sf9 cells.

**ERK Phosphorylates GRK2 in Vitro**—To determine whether ERK may indeed be the enzyme responsible for phosphorylating GRK2 in cells, the ability of purified ERK1 to phosphorylate unphosphorylated or phosphorylated GRK2 was examined in vitro. ERK1 phosphorylated unphosphorylated but not phosphorylated GRK2. However, this phosphorylation occurred only when heparin, an inhibitor of GRK2, was present in the phosphorylation reaction (Fig. 3A, compare lanes 5 and 6). These results demonstrate that ERK1 can phosphorylate GRK2 in vitro and that this phosphorylation occurs on Ser670, because GRK2 phosphorylated at this site is not a substrate for ERK1 (Fig. 3A, compare lane 6, upper and lower panels).

There are several potential explanations for the requirement of heparin in these in vitro phosphorylation reactions. GRK2 may phosphorylate and inactivate ERK1. ERK1-mediated GRK2 phosphorylation would thus be predicted to occur only when GRK2 activity is inhibited, i.e. in the presence of heparin. Alternatively, ERK1-mediated GRK2 phosphorylation may be conformation-dependent. Heparin may mimic a natural ligand of GRK2 that upon binding exposes Ser670. GRK2 has no effect on ERK1 activity as assessed by the ability of ERK1 to phosphorylate myelin basic protein in the presence or absence of GRK2 (data not shown). Additionally, ERK1 fails to phosphorylate GRK2 in the presence of heparin when GRK2 is denatured. These results suggest that the conformation of GRK2 is critical for ERK1-mediated phosphorylation (data not shown).

The nature of the putative ligand required for ERK-mediated GRK2 phosphorylation in cells remains to be elucidated. Known regulators of GRK2 activity including, Gβγ and/or PIP2, phosphatidylserine, and Ca2+/calmodulin failed to support ERK1-mediated GRK2 phosphorylation (data not shown).

The ERK1-mediated phosphorylation of GRK2 observed in vitro would be predicted to alter the functional properties of this enzyme if ERK1 phosphorylates Ser670 and phosphorylation at this site is responsible for attenuating the Gβγ sensitivity of this enzyme. Unphosphorylated GRK2 incubated in the presence of heparin in either the presence or absence of ERK1 was subjected to gel filtration to remove heparin, and its ability to phosphorylate rhodopsin in the presence of varying concentrations of Gβγ was examined. As shown in Fig. 3B ERK1-mediated phosphorylation of GRK2 dramatically impairs GRK2 activity and sensitivity to Gβγ activation. These results again support the assignment of Ser670 as the phosphorylated residue in GRK2 and suggest that under the appropriate conditions ERK1 is capable of mediating this phosphorylation event.

The requirement of heparin in the in vitro ERK1 phosphorylation assays raises the question of whether this kinase is responsible for phosphorylating GRK2 in cells. To examine a potential role for ERKs in phosphorylating and regulating GRK2 activity in cellular systems, we examined the effect of modulating ERK activity on GRK2-mediated β2AR phosphorylation.

**Modulating ERK Activity in Cells Regulates GRK2 Function**—HEK293 cells transiently transfected with FLAG-tagged β2AR, and GRK2 were additionally transfected with either a dominant-negative or a constitutively active form of ERK kinase-1 (MEK1). MEK1 is a dual specificity threonine/tyrosine kinase that phosphorylates and activates ERK1 and -2. Transfection of dominant-negative (MEK(K/A)) or constitutively active (MEK(DD)) forms of MEK thus regulates ERK activity in cells. Transfected HEK293 cells were labeled with [32P]P, and the ability of GRK2 to phosphorylate β2AR assessed by immuno-
Regulation of GRK2 Activity by ERKs

The discovery that GRK2 exists as a phosphoprotein in cells provides a striking analogy between GRK2 and β-arrestin1 (15). β-Arrestin1 binds to GRK-phosphorylated GPCRs and serves to uncouple the phosphorylated receptor from G proteins and to target it to clathrin-coated pits for internalization. β-Arrestin1, like GRK2, exists as a phosphoprotein in cells (13). Indeed, ERK has recently been shown to phosphorylate β-arrestin1 in cells (15). Dephosphorylation of β-arrestin1 occurs at the plasma membrane following agonist occupancy of GPCRs and is required for the high affinity interaction of β-arrestin1 and clathrin (13). Thus, two proteins required for mediating the internalization of agonist-occupied GPCRs, GRK2 and β-arrestin1, are ERK substrates. Engagement of the clathrin-coated pit endocytosis machinery has been shown to be required for ERK activation by the β2-AR (3). By phosphorylating and regulating the activity of two key players in this internalization process, ERK can thus be envisioned as participating in a negative feedback regulatory loop.

It is of interest to note that ERK phosphorylation at Ser412 is specific to β-arrestin1, because this residue is not conserved in other members of the arrestin family. Similarly Ser670 of GRK2 is not conserved in GRK3. It will be of interest to determine whether phosphorylation by ERK is a regulatory mechanism specific to GRK2 and β-arrestin1 or if other members of these protein families are regulated by ERK phosphorylation. The observation that β-arrestin1 is dephosphorylated at the plasma membrane following agonist occupancy of GPCRs suggests, by analogy, that a similar mechanism for dephosphorylating GRK2 may exist. These two proteins may be dephosphorylated by the same phosphatase. Although several questions remain concerning the exact mechanism of ERK-mediated GRK2 phosphorylation, the elucidation of a potential role for ERK in regulating this enzyme provides novel insights into the mechanisms controlling GPCR-mediated signal transduction.

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