MAPK-dependent Degradation of G Protein-coupled Receptor Kinase 2*

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G protein-coupled receptor kinase 2 (GRK2) is a key modulator of G protein-coupled receptors (GPCR). Altered expression of GRK2 has been described to occur during pathological conditions characterized by impaired GPCR signaling. We have reported recently that GRK2 is rapidly degraded by the proteasome pathway and that β-arrestin function and Src-mediated phosphorylation are involved in targeting GRK2 for proteolysis. In this report, we show that phosphorylation of GRK2 by MAPK also triggers GRK2 turnover by the proteasome pathway. Modulation of MAPK activation alters the degradation of transfected or endogenous GRK2, and a GRK2 mutant that mimics phosphorylation by MAPK shows an enhanced degradation rate, thus indicating a direct effect of MAPK on GRK2 turnover. Interestingly, MAPK-mediated modulation of wild-type GRK2 stability requires β-arrestin function and is facilitated by previous phosphorylation of GRK2 on tyrosine residues by c-Src. Consistent with an important physiological role, interfering with this GRK2 degradation process results in altered GPCR responsiveness. Our data suggest that both c-Src and MAPK-mediated phosphorylation would contribute to modulate GRK2 degradation, and put forward the existence of new feedback mechanisms connecting MAPK cascades and GPCR signaling.

G protein-coupled receptors (GPCR) detect a broad spectrum of extracellular signals at the plasma membrane, thereby modulating key cellular functions as diverse as growth, differentiation, inflammation, or neurotransmission (1, 2). Agonist-occupied receptors promote the activation and dissociation of heterotrimeric G proteins into α and βγ subunits, both of which regulate a wide variety of effector systems. In addition, agonist stimulation also leads to the deactivation of GPCR signaling (desensitization) by triggering receptor phosphorylation by specific G protein-coupled receptor kinases (GRKs) and binding of the cytosolic proteins β-arrestins to the phosphorylated receptor (3, 4). GRK2 is a ubiquitous member of the GRK family, which has been shown to modulate a variety of GPCRs (5, 6).

The same regulatory molecules that contribute to receptor uncoupling from G proteins also regulate GPCR endocytosis, intracellular trafficking, and resensitization and participate in the modulation of mitogen-activated protein kinase (MAPK) cascades by GPCR (7). Thus, β-arrestins mediate the recruitment of clathrin and β2-adaptin to allow for receptor internalization and also act as scaffold molecules by bridging receptors with signaling proteins such as c-Src, thus facilitating the activation of the ERK/MAPK cascade by GPCR (8, 9). In addition, the isoforms of β-arrestins can directly interact with components of two different MAPK cascades bringing these molecules into close proximity with the receptor complex (10, 11). On the other hand, GRK2 has been shown to interact with the ARF modulator GIT, with phosphatidylidyinositol 3′-OH kinase γ, and the G protein αs subunit (12) and to phosphorylate non-receptor substrates such as tubulin, synucleins, or phosphducin (13), thus also extending the cellular functions of GRK2 beyond GPCR desensitization.

The key role that GRKs and β-arrestins play in GPCR signaling and modulation suggests that the overall activity of GPCRs would be strongly dependent on the cellular complement and functionality of these proteins. Consistent with this idea, the altered expression of GRK2 described in several pathological conditions such as hypertension (14), congestive heart failure (15), or rheumatoid arthritis (6) has been correlated with impaired GPCR signaling in these situations. GRK2 activity and subcellular distribution are tightly regulated by interactions with Gβγ subunits, lipids, agonist-activated receptors, anchoring proteins and calmodulin, or phosphorylation by other kinases (reviewed in Refs. 16–18). On the other hand, the mechanisms that govern GRK2 cellular levels and that may explain its alterations in physiological and pathological conditions have begun to be addressed recently. In this regard, we have reported recently (19) that GRK2 is degraded by the proteasome pathway and that kinase turnover was enhanced upon GPCR stimulation. Moreover, we have shown that β-arrestin function and c-Src activity are involved in GRK2 proteolysis. Agonist-dependent binding of β-arrestin to GPCRs allows for the recruitment of c-Src, leading to phosphorylation of GRK2 on tyrosine residues and its targeting for degradation (20). However, the occurrence of additional pathways that may control GRK2 stability was also suggested by some of our data (20).

We and others (21, 22) have described that GPCR activation promotes the presence of active MAPK and GRK2 in the same multimolecular complex and that MAPK phosphorylates GRK2. In this report, we show that MAPK-mediated GRK2 phosphorylation triggers GRK2 degradation in a process that appears to be facilitated when GRK2 is previously tyrosine-
phosphorylated. Our data indicate that both c-Src and MAPK pathways would contribute to modulate GRK2 protein stability by promoting proteasome-dependent GRK2 degradation, and put forward new functional regulatory relationships between MAPK cascades and GPCR signaling.

EXPERIMENTAL PROCEDURES

Materials and Plasmids—HEK-293, COS-7, and Jurkat T cells were from the American Type Culture Collection (Manassas, VA). Culture media and LipofectAMINE were from Invitrogen. Protein A-Sepharose and isopropenol were purchased from Sigma. [35S]Methionine and [35S]Cysteine labeling mixture was from PerkinElmer Life Sciences. Horseradish peroxidase-conjugated IgG and IgM were from Roche Diagnostics and Bio-Rad, respectively. Site-directed mutagenesis kit (Stratagene) using the following PCR method. Thus, this mutant was generated by sequentially using the external primers upstream (nucleotides 1725–1785) and downstream (nucleotides 2199–2220) of the mutation in combination with the mutant oligonucleotides 5′-CCGCCGCCGGCGCCGCTCGAG-CTCGAGGAAAG-3′ and its pair 5′-GACGGGGTCGCGCGGCTTGTTCTTCATCTTT-CATCTTT-3′. The amplified product was subcloned into ApaI-Xhol sites in pcDNA3. The GRK2–S670A mutant was generated using the “Quick Change Site-directed mutagenesis kit” (Stratagene) using the following primers 5′-CCGCCGCCGGCGCCGCTCGAGCTGACCGGAAAG-3′ and its pair 5′-GACGGGGTCGCGCGGCTTGTTCTTCATCTTT-CATCTTT-3′. Finally, the double mutants GRK2-Y13F/2AR, receptor activation was triggered by 10 μM isoproterenol (PeproTech). The protein aPKC (PKCζ), and MEK (MEKζ) were kindly provided by Dr. J. Moscat (Centro de Biología Molecular, Madrid, Spain). The eDNA of constitutively active Ras (H-Ras V-12) was obtained from Dr. M. Serrano (Centro Nacional de Biotecnología, Madrid, Spain). The source of other expression plasmids and materials was reported previously (19, 20). All other reagents were of the highest grade commercially available.

Site-directed Mutagenesis of GRK2—Specific GRK2 mutation at serine 670 to aspartic acid (GRK2–S670D) was performed by using a “bridge” PCR method. Thus, this mutant was generated by sequentially using the external primers upstream (nucleotides 1725–1785) and downstream (nucleotides 2199–2220) of the mutation in combination with the mutant oligonucleotides 5′-CCGCCGCCGGCGCCGCTCGAG-CTCGAGGAAAG-3′ and its pair 5′-GACGGGGTCGCGCGGCTTGTTCTTCATCTTT-CATCTTT-3′. The amplified product was subcloned into ApaI-Xhol sites in pcDNA3. The GRK2–S670A mutant was generated using the “Quick Change Site-directed mutagenesis kit” (Stratagene) using the following primers 5′-CCGCCGCCGGCGCCGCTCGAGCTGACCGGAAAG-3′ and its pair 5′-GACGGGGTCGCGCGGCTTGTTCTTCATCTTT-CATCTTT-3′. Finally, the double mutants GRK2-Y13F/2AR, receptor activation was blocked with the antagonist betaxolol (10 μM; Calbiochem) were added 90 min prior to metabolic labeling or cell lysis. In some experiments, endogenous CXC4 receptors in Jurkat cells maintained in 1% serum for 18 h were activated with the chemokine SDF-1α (100 nM for 5 min, PeproTech). The pro tease inhibitors MG132 (10 μM; Bioval) and ALLN (50 μM; Sigma) were added 90 min before labeling and maintained during the chase period.

Immunoprecipitation and Western Blot Analysis—Lysates were harvested and lysed in RIPA buffer, and cellular extracts were immunoprecipitated with the specific GRK2 polyclonal antibody AbFP1 as reported (20). Immunoprecipitates were resolved in 10% SDS-PAGE and gels either subjected to fluorography (pulse-chase experiments) or transferred to nitrocellulose membranes to be probed with an antiphospho-tyrosine monoclonal antibody (PY99-horseradish peroxidase; Santa Cruz Biotechnology) and then reprobed after stripping with AbFP1. In some experiments, the presence of GRK2 phosphorylated on serine 670 in the immunoprecipitates was tested by using an specific anti-phosphoserine polyclonal antibody (pS670 anti-GRK2 antibody from BIO- SOURCE International). MAPK activation was detected in cellular lysates by using a phospho-p42/p44 MAPK (Thr-202/Tyr-204) polyclonal antibody (1:500, New England Biolabs). The same blots were subsequently reprobed with a p42/p44 MAPK polyclonal antibody (Calbiochem) to normalize MAPK activation to whole MAPK protein levels. Lysate aliquots were taken to assess protein overexpression of the different wild-type and mutant constructs as described (20). When indicated, actin expression was determined by using a polyclonal antibody (Santa Cruz Biotechnology). Blots were developed using a chemiluminescent method (ECL, Amersham Biosciences). Band density was quantitated by laser densitometric analysis.

RESULTS

Based on previous reports (21, 22) that showed agonist-mediated association between GRK2 and MAPK as well as GRK2 phosphorylation by the latter kinase, we explored whether modulation of the ERK/MAPK pathway could have any effect on GRK2 degradation. To this end, basal and isoproterenol-stimulated GRK2 turnover was determined by pulse-chase experiments in HEK-293 cells under conditions that would impair MAPK activation. Fig. 1 shows that the enhanced GRK2 degradation induced by agonist was strongly inhibited in the presence of the MEK inhibitor UO126 or upon co-transfection of a dominant-negative ERK1 construct. These data indicated that MAPK activation is involved in agonist-induced GRK2 proteolysis. Interestingly, inhibition of MAPK activation also leads to a slower GRK2 turnover under unstimulated conditions, suggesting that even in the absence of β2-adrenergic receptor (β2AR) agonists, different endogenousGPCRs and/or the basal activity of overexpressed β2AR (20) are modulating GRK2 stability through a mechanism also involving MAPK stimulation. Consistent with a role for basal β2AR activity in GRK2 turnover, the degradation of GRK2 in cells co-expressing β2AR was clearly delayed upon treatment with the antagonist betaxolol (93 ± 4.5% of GRK2 remaining after 1 h of chase with betaxolol versus 51 ± 10% without, data not shown).

To explore further the involvement of MAPK in GRK2 degradation, we examined whether the expression of constitutively active upstream activators of the MAPK cascade would affect GRK2 turnover. HEK-293 cells were transiently transfected with GRK2 and a mutant construct of Raf (RafA) lacking an NH2-terminal regulatory domain (23), thus promoting a robust MAPK activation without altering MAPK protein levels (data not shown). The sole expression of this mutant notably increases GRK2 degradation as assessed by pulse-chase experiments (31.6 ± 3.8% of kinase remaining after 1 h compared with 51 ± 10% in control conditions) (Fig. 2A). Moreover, the expression of an active pCKCζ mutant (pCKCζ+) that stimulates MAPK activity in a Raf-dependent manner (24) also enhances the GRK2 degradation rate (32.5 ± 0.7% of kinase remaining after 1 h of chase) (Fig. 2B). Similar patterns of GRK2 decay were also detected by expressing a constitutively active Ras mutant (data not shown). The augmented component of GRK2 decay was also detected by expressing a constitutively active Ras mutant (data not shown). The augmented component of GRK2 decay was also detected by expressing a constitutively active Ras mutant (data not shown). The augmented component of GRK2 decay was also detected by expressing a constitutively active Ras mutant (data not shown). The augmented component of GRK2 decay was also detected by expressing a constitutively active Ras mutant (data not shown). The augmented component of GRK2 decay was also detected by expressing a constitutively active Ras mutant (data not shown). The augmented component of GRK2 decay was also detected by expressing a constitutively active Ras mutant (data not shown). The augmented component of GRK2 decay was also detected by expressing a constitutively active Ras mutant (data not shown).
MAPK-dependent GRK2 Degradation

Fig. 1. MAPK activity modulates GRK2 degradation. HEK-293 cells transiently expressing GRK2 and β2AR were co-transfected with a dominant-negative ERK construct (dnERK1) or empty vector (A) or pretreated with the MEK inhibitor UO126 or vehicle (B) as detailed under “Experimental Procedures.” GRK2 turnover was determined by pulse-chase experiments in the presence or absence of the β-agonist isoproterenol (10 μM) for the indicated times. Labeled proteins were immunoprecipitated with the specific anti-GRK2 antibody AbFP1 and resolved by SDS-PAGE followed by fluorography and densitometry as described under “Experimental Procedures.” The inhibition of MAPK activity by dnERK1 expression or UO126 treatment was confirmed by immunoblot analysis of lysate aliquots as detailed under “Experimental Procedures.” Data are the mean ± S.E. of 3–4 experiments performed in duplicate.

Fig. 2. Stimulation of the MAPK pathway increases GRK2 degradation. A and B, HEK-293 cells were transiently transfected with GRK2 alone or in the presence of constitutively active Raf (Raf*) and PKCζ (PKCζ*) constructs, and GRK2 turnover was determined by pulse-chase experiments as described under “Experimental Procedures” and Fig. 1. C, the expression of a dominant-negative mutant of MAPK (dnERK1) inhibits Raf*-induced increase in GRK2 turnover in cells transfected with GRK2 and Raf*. Data in A and C are the mean ± S.E. of 3–4 experiments performed in triplicate. Representative gel fluorographies are shown in B. The expected changes in MAPK activity in the different experimental conditions were confirmed as in Fig. 1 (data not shown).

(not shown), markedly impairs endogenous GRK2 degradation as assessed by pulse-chase experiments. Consistently, steady-state GRK2 levels detected by immunoblot analysis are increased in Jurkat cells in these conditions (Fig. 3B). Such increase is specific, because the amount of other cellular proteins remains unaltered (Fig. 3B, lower panel).

The effect of MAPK on GRK2 degradation could be a consequence of the direct phosphorylation of GRK2 by MAPK or an indirect effect caused by MAPK-mediated phosphorylation of other proteins. In this regard, it has been reported previously...
MAPK-dependent GRK2 Degradation

that GRK2 is phosphorylated in vivo at the MAPK consensus phosphorylation site Ser-670 (22). To address the potential role of direct MAPK phosphorylation of GRK2 on its degradation, we generated mutants at Ser-670 to both mimic MAPK phosphorylation (S670D) or to prevent it (S670A). As shown in Fig. 4, the turnover of the S670D mutant transiently expressed in HEK-293 cells was clearly increased as compared with wild-type GRK2, and its degradation rate was very similar to that of wild-type GRK2 (Fig. 4) and takes place by the proteasome pathway, because specific inhibitors efficiently blocked proteolysis (not shown). However, the marked increase in wild-type GRK2 degradation that takes place in the presence of β-adrenergic agonists is not observed with the GRK2-S670A mutant (Fig. 4). These results suggested that GRK2 phosphorylation by MAPK plays a role in accelerating GRK2 degradation, although such covalent modification is not strictly required for proteasomal targeting of the kinase, pointing to the occurrence of alternative and independent pathways leading to GRK2 degradation.

We next analyzed in more detail the mechanisms underlying GRK2-S670A turnover. GPCR can sequentially activate c-Src and MAPK, and GRK2 can be phosphorylated by both kinases in an agonist-dependent way. Since we have recently shown (20) that c-Src-mediated phosphorylation is involved in GRK2 proteolysis, we explored the potential role of tyrosine phosphorylation on GRK2-S670A degradation. Thus, tyrosine residues critical for c-Src phosphorylation (20) were mutated to phenylalanine in the GRK2-S670A construct. Interestingly, the turnover of this combined mutant was severely impaired as compared with GRK2-S670A or wild-type GRK2 (81.6 ± 5.5% of protein remaining after 1 h of chase) (Fig. 5A) and is more similar to the pattern described for the Y13F/Y86F/Y92F mutant (84 ± 6% of labeled protein remaining at 1 h of chase). These data indicate that when the potential modulation of GRK2 degradation by MAPK-phosphorylation is abolished, the proteolysis takes place through a tyrosine phosphorylation-dependent mechanism, suggesting the involvement of different regulatory processes in GRK2 degradation. Interestingly, mutation of the residues critical for c-Src phosphorylation in the S670D mutant (GRK2-Y13F/Y86F/Y92F-S670D) has no effect on its turnover (Fig. 5B). Moreover, co-transfection with a catalytically inactive mutant of the kinase domain of c-Src (SH1-Kp), which does block wild-type GRK2 degradation (20), does not alter the rate of degradation of the GRK2-S670D mutant (Fig. 5B). These results would suggest that once GRK2 was not significantly retarded when compared with that of wild-type GRK2 (Fig. 4) and takes place by the proteasome pathway, because specific inhibitors efficiently blocked proteolysis (not shown). However, the marked increase in wild-type GRK2 degradation that takes place in the presence of β-adrenergic agonists is not observed with the GRK2-S670A mutant (Fig. 4). These results suggested that GRK2 phosphorylation by MAPK plays a role in accelerating GRK2 degradation, although such covalent modification is not strictly required for proteasomal targeting of the kinase, pointing to the occurrence of alternative and independent pathways leading to GRK2 degradation.

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FIG. 3. Modulation of endogenous GRK2 degradation by MAPK pathway inhibitors. A, the turnover of endogenous GRK2 in Jurkat T cells was assessed by pulse-chase experiments as described under “Experimental Procedures” in the presence of a MEK inhibitor (PD98059, 10 μM) or vehicle (Me2SO). Data are the mean ± S.E. of 3 experiments performed in triplicate. B, effect of inhibition of the MAPK pathway on GRK2 expression levels in Jurkat T cells. Cells were incubated for the time indicated in the presence or absence (control conditions) of the MEK inhibitor PD98059, as detailed under “Experimental Procedures,” and the steady-state levels of GRK2 were determined by immunoblot analysis of cell lysates. A control (lanes C), actin and Erk levels as well as the activation state of MAPK were determined in the same blots as detailed under “Experimental Procedures.” Data are representative of two independent experiments.

FIG. 4. Phosphorylation of GRK2 by MAPK directly modulates its degradation. A HEK-293 cells were transiently transfected with wild type GRK2 or the MAPK phosphorylation site mutants S670A or S670D, and their stability, in the absence of presence of the agonist isoproterenol (ISO) as indicated, was determined by pulse-chase experiments as detailed under “Experimental Procedures” and Fig. 1. Data are mean ± S.E. of 3–4 independent experiments performed in triplicate. Representative fluorographies are shown for the basal degradation of GRK2 mutants.
is phosphorylated by MAPK, its degradation could take place independently of Src activity.

Overall, these data suggest that c-Src and MAPK-mediated phosphorylation target GRK2 to the proteasome pathway independently. Once GRK2 is phosphorylated by MAPK, it proceeds for degradation, and tyrosine phosphorylation is dispensable. If GRK2 cannot be phosphorylated by MAPK, proteolysis takes place through a tyrosine phosphorylation-dependent pathway. In fact, both wild-type GRK2 and S670A mutant are efficiently tyrosine-phosphorylated upon β2AR stimulation (data not shown). However, some kind of functional relationship appears to occur between these two turnover mechanisms. Fig. 5C shows that the expression of constitutively active Raf does not increase the degradation rate of the Y13F/Y86F/Y92F GRK2 mutant nor does the presence of the MEK inhibitor PD98059 further retard the GRK2 Y13F/Y86F/Y92F turnover. These data suggest that, for wild-type GRK2, MAPK phosphorylation might be preferentially modulating the stability of the fraction of the GRK2 cellular pool previously phosphorylated on tyrosine residues. Experiments of stimulation of endogenous CXCR4 receptors in Jurkat cells are consistent with this sequential model. Activation of CXCR4 receptors leads to both Src and MAPK stimulation, although activation of the latter is not dependent on tyrosine kinase activity (data not shown). We observed that CXCR4 stimulation promotes the rapid phosphorylation of endogenous GRK2 on serine 670, as assessed with a phosphoserine 670-specific antibody (P-GRK2S670). After stripping, the presence of similar quantities of GRK2 in the immunoprecipitates was analyzed in the same gel with the AbFP1 polyclonal antibody. Data in the different panels are either the mean ± S.E. of three independent experiments performed in duplicate or representative of three independent experiments. IB, immunoblot.

**Fig. 5.** Both tyrosine and MAPK phosphorylation-dependent pathways affect GRK2 degradation. A, HEK-293 cells were transfected with either wild-type GRK2 or the mutant S670A or Y13F/Y86F/Y92F-S670A GRK2 constructs, and their degradation rate was determined after 1 h of chase as described under “Experimental Procedures” and Fig. 1. B, effect of blocking Src-mediated tyrosine phosphorylation on the turnover of GRK2-S670D mutants. HEK-293 cells were transiently transfected with a GRK2-Y13F/Y86F/Y92F-S670D mutant (upper panel) or cotransfected with GRK2-S670D and empty vector (−) or the dominant-negative SH1KD c-Src mutant. C, effect of MAPK modulation on the degradation of a GRK2 mutant unable to be phosphorylated by c-Src. HEK-293 cells were transfected with the GRK2-Y13F/Y86F/Y92F mutant alone or with constitutively active Raf (Raf*), and its turnover was analyzed as in previous figures in the absence (control and Raf* conditions) or presence of a MEK inhibitor (PD98059). Similar degradation patterns are observed in all situations. D, MAPK-mediated phosphorylation of endogenous GRK2 in serine 670 is blocked in the presence of a c-Src inhibitor. Jurkat cells were treated with the indicated combinations of the CXCR4 agonist SDF1-α, the MEK inhibitor PD98059, and the c-Src inhibitor PP2 as detailed under “Experimental Procedures.” Immunoprecipitates (IP) of GRK2 were analyzed with a phosphoserine 670-specific antibody (P-GRK2S670). After stripping, the presence of similar quantities of GRK2 in the immunoprecipitates was analyzed in the same gel with the AbFP1 polyclonal antibody. Data in the different panels are either the mean ± S.E. of three independent experiments performed in duplicate or representative of three independent experiments. IB, immunoblot.
that the kinase-dead GRK2-K220R is barely degraded when expressed in cells. This is due to its inability to promote the recruitment of β-arrestin and associated molecules such as c-Src to the vicinity of the receptor, because β-arrestin overexpression can "rescue" GRK2-K220R turnover (20). Interestingly, overexpression of Raf* does not promote GRK2-K220R proteolysis (Fig. 6A), supporting the hypothesis that additional factors are required for MAPK-induced GRK2 turnover. Therefore, we explored whether co-transfection of β-arrestin1 with GRK2-K220R and Raf* had any effect on the degradation of this mutant. The presence of Raf* causes a clear additional increase in the turnover of GRK2-K220R promoted by β-arrestin overexpression (Fig. 6A, lower panel). In other experiments, HEK-293 cells were co-transfected with Raf* and wild-type GRK2 in the presence of different β-arrestin1 mutants (Fig. 6B). Expression of wild-type β-arrestin1 had no effect on the enhanced GRK2 degradation promoted by Raf*, indicating that endogenous β-arrestin levels in these cells are sufficient to support GRK2 degradation as demonstrated previously (20).

On the contrary, expression of β-arrestin-S412D (which is unable to recruit c-Src, Ref. 18) blocked the degradation induced by Raf*, whereas β-arrestin-S412A (which binds efficiently to Src) has the same effect as wild-type β-arrestin.

Overall, our results demonstrate that MAPK-mediated control of GRK2 turnover normally requires phosphorylation of GRK2 on tyrosine residues by c-Src and the involvement of β-arrestin as a protein adaptor. This is in agreement with the ability of β-arrestin to recruit c-Src (18) and to assemble signaling complexes involving Raf and Erk (10), and possibly additional proteins needed to promote GRK2 degradation (see "Discussion").

The complex mechanisms of control of GRK2 stability suggest that such tight modulation could have a role in the regulation of GPCR signaling. Therefore, we explored whether modifications in GRK2 levels and turnover rate may alter subsequent cellular responses to GPCR agonists. In a first set of experiments, we found that chronic agonist challenge of endogenous βAR in HEK-293 cells clearly down-regulates endogenous GRK2 levels (0.56 ± 0.0 of cellular GRK2 complement after isoproterenol treatment as compared with control cells incubated with vehicle, Fig. 7A). Interestingly, MAPK activation in response to LPA was clearly enhanced in those cells with lower GRK2 levels as a result of isoproterenol pretreatment (Fig. 7A, right panels, 4.2 ± 0.4-fold ERK2 activation over unstimulated conditions in pretreated cells versus 2.5 ± 0.5-fold activation in control cells, data from three independent experiments with triplicate determinations). Therefore, a reduction in the pool of GRK2 in an endogenous system appears to favor subsequent responsiveness even to other GPCR. These results suggested that agonist-stimulated degradation of GRK2 might contribute to preserve GPCR responsiveness in physiological conditions characterized by sustained GPCR stimulation. The importance of an adequate control of GRK2 turnover in GPCR signaling is also supported by similar experiments performed in COS-7 cells overexpressing β2AR and either wild-type GRK2 or the GRK2-S670A mutant (Fig. 7B). As observed
in the endogenous system, wild-type GRK2 protein levels declined upon chronic \( \beta_2 \text{AR} \) stimulation (0.63 ± 0.08 of kinase levels as compared with control conditions). However, no significant changes in GRK2-S670A protein expression were observed in response to isoproterenol, consistent with its deficient agonist-induced degradation. When subsequent activation of endogenous LPA receptors was tested, MAPK activation was clearly impaired as compared with wild-type conditions in pretreated cells expressing the GRK2-S670A mutant. In these experimental conditions, we do not observe in wild-type GRK2-overexpressing cells the additional increase in MAPK activation in response to LPA as consequence of isoproterenol pretreatment that is apparent in an endogenous system. However, the clear disparity in MAPK response between wild-type GRK2 and GRK2-S670A-expressing cells is likely due to stability differences, because distal components of the MAPK cascade are not altered, as epidermal growth factor promotes similar MAPK activation in cells expressing either wild-type or mutant GRK2 (data not shown). Taken together, these observations strongly suggest that changes in GRK2 turnover are important for the fine-tuning of signaling.

**DISCUSSION**

In this report we show that MAPK phosphorylation regulates the degradation of GRK2, thus putting forward a new functional relationship between MAPK cascades and GPCR signaling. Several lines of evidence support the notion that MAPK activation promotes GRK2 turnover. In transfected cells, direct stimulation of the ERK/MAPK pathway at different levels markedly increases GRK2 proteolysis. On the other hand, a GRK2-S670A mutant shows retarded degradation in response to GPCR activation, consistent with a role for MAPK in accelerating GRK2 proteolysis. This mechanism would also explain previous observations of our laboratory showing that the dynamin K44A mutant inhibited agonist-induced GRK2 degradation in a receptor internalization-independent manner (20).
The effect of the dynamin mutant would now be adequately explained by its reported effect on the activation of MAPK cascades (25).

The control of GRK2 stability emerges as a complex process involving different signaling pathways. We have reported recently (20) that recruitment of β-arrestin and c-Src-mediated GRK2 phosphorylation on tyrosine residues are critical signals that trigger GRK2 degradation through the proteasome pathway. A key question is how Src and MAPK-dependent mechanisms contribute to GRK2 turnover. In order to address this issue, we have investigated how different experimental conditions affect the turnover of a variety of GRK2 mutants. These include proteins lacking residues critical for MAPK (GRK2-S670A) or c-Src (GRK2 Y13F/Y86F/Y92F)-mediated phosphorylation, a mutant that mimics permanent phosphorylation by MAPK (GRK2–S670D), or combinations of those (GRK2 Y13F/Y86F/Y92F-S670A; GRK2 Y13F/Y86F/Y92F-S670D).

It is worth noting that, although the agonist-induced turnover of the GRK2 S670A mutant is significantly retarded with respect to the wild-type kinase, the basal degradation is not blocked. This would suggest that MAPK-mediated modulation is relevant for agonist-promoted turnover, although additional signals can trigger GRK2 proteolysis. The alternative pathway for GRK2-S670A degradation appears to be dependent on tyrosine phosphorylation, because the turnover of the combined Y13F/Y86F/Y92F-S670A mutant is severely impaired. On the contrary, when permanent phosphorylation by MAPK is mimicked by the S670D mutation, the protein rapidly proceeds to degradation, even if Src activity is blocked or if tyrosine phosphorylation of the mutant is hampered by replacing the critical tyrosine residues (GRK2-Y13F/Y86F/Y92F-S670D mutant). Overall, these data suggest that either tyrosine or MAPK-dependent phosphorylation can independently trigger GRK2 degradation by the proteasome pathway. However, our results also indicate the occurrence of a “facilitatory” role of GRK2 tyrosine phosphorylation for the subsequent MAPK-dependent control of wild-type GRK2 turnover. The degradation of a GRK2 mutant unable to be phosphorylated by Src, such as Y13F/Y86F/Y92F, is not enhanced nor inhibited by activators or blockers of MAPK cascades, respectively. It could be hypothesized that tyrosine-phosphorylated GRK2 is a better substrate for MAPK. In fact, it has been reported that GRK2 must undergo some kind of conformational change to be efficiently phosphorylated by MAPK (22). In line with this, we find that rapid agonist-induced phosphorylation of endogenous GRK2 in serine 670 is blocked in the presence of a c-Src inhibitor (Fig. 5D).

It is worth noting that β-arrestin function appears to play a central role in both Src (20) and MAPK-dependent GRK2 degradation processes. In addition to recruiting c-Src (8) and thus allowing for GRK2 phosphorylation on tyrosine residues (20, 26), β-arrestin has been reported to assemble key components

![Proposed model for the regulation of GRK2 turnover by different signaling pathways.](http://www.jbc.org/)

Receptor activation would trigger the sequential recruitment of GRK2, β-arrestin, c-Src, and probably other proteins, leading to GRK2 phosphorylation on tyrosine residues. Such covalent modification promotes “per se” GRK2 degradation by the proteasome pathway. An additional pathway for proteasomal GRK2 degradation is promoted upon MAPK-mediated modification of tyrosine-phosphorylated GRK2 in such cellular context. Mimicking constitutive GRK2 phosphorylation by MAPK in the S670D mutant relieves the requirement for previous GRK2 phosphorylation by c-Src. The proposed site of action of the different mutants and pharmacological inhibitors used in our studies is indicated.
of the MAPK cascade (Raf, MEK, and ERK) in the vicinity of activated GPCR (9, 10). The scaffold function of β-arrestin would contribute to the spatial and sequential organization of the multimolecular complexes where the functional relationships among Src, MAPK, and GRK2 could take place. Moreover, β-arrestin has been reported recently (27) to interact with Mdm2 and other as yet unidentified ubiquitin ligases involved in β-AR and β-arrestin ubiquitination. By recruiting these or other ubiquitin-protein isopeptide ligases, β-arrestin may also contribute to link Src and/or MAPK-phosphorylated GRK2 to the degradation machinery.

The scheme in Fig. 8 depicts our current working model for the molecular mechanisms of GRK2 degradation through the proteasome pathway. Receptor activation would promote the sequential recruitment of GRK2, β-arrestin, and c-Src to the GPCR complex, resulting in tyrosine phosphorylation of GRK2 (26). This process can be inhibited at several steps, leading to impaired GRK2 degradation. GRK2-K220R would bind to the receptor but not promote the sequential binding of β-arrestin and c-Src (what can be rescued by β-arrestin overexpression); phosphorylation of GRK2 on tyrosine residues can be inhibited by specifically blocking β-arrestin/c-Src interaction with either β-arrestin S412D (8) or the SH1KD constructs (28), by pharmacological tyrosine kinase inhibitors (not shown in the scheme), or GRK2 mutants lacking critical residues for c-Src-mediated phosphorylation (20). Tyrosine phosphorylation of GRK2 would be sufficient for triggering kinase proteolysis through the proteasome pathway. In addition, it would facilitate GRK2 phosphorylation by MAPK and a subsequent, additional targeting route to the proteasome. Both degradation pathways would contribute to GRK2 turnover upon GPCR stimulation, consistent with the fact that this process is retarded in the presence of MAPK cascade inhibitors or in the GRK2-S670A mutant. Although previous phosphorylation by c-Src is normally required, mimicking GRK2 phosphorylation by MAPK as in the S670D mutants would directly lead to rapid degradation through the latter pathway independent of phosphorylation by c-Src. Such a model for the control of GRK2 stability would allow for the fine regulation of GRK2 turnover rates, integrating inputs such as GPCR stimulation and the activity of cytosolic tyrosine kinases and MAPK cascades.

An increasing number of reports show that protein phosphorylation functions as a regulatory label for triggering proteasomal degradation (29) of a variety of signaling proteins. Phosphorylation on tyrosine residues can trigger ubiquitination and degradation of the epidermal growth factor receptor, Src-kinases, E-cadherin or JAK-2 by promoting their interaction with specific ubiquitin-protein isopeptide ligase adaptors (30–33). Recent examples of MAPK phosphorylation-dependent proteasomal degradation include the inducible cAMP early repressor ICER, the TAL1/SC2 transcription factor, cell cycle inhibitors, or the upstream kinase Ste11 in yeast (34–37). The identification of proteins able to interact specifically with tyrosine or MAPK-phosphorylated GRK2 that could help target the kinase for degradation is currently underway in our laboratory.

The existence of these complex mechanisms of control of GRK2 stability appears to play a relevant physiological role as a feedback mechanism that would modulate receptor responsiveness, limiting the extent of desensitization upon chronic receptor stimulation under physiological and pathological conditions. As shown in this report, persistent βAR activation decreases GRK2 cellular levels and allows an enhanced MAPK activation in response to other GPCR agonist such as LPA. However, when such a normal decrease in GRK2 levels upon GPCR stimulation is impaired by suppressing the MAPK pathway component in the GRK2-S670A mutant, subsequent activation of the MAPK cascade by LPA is clearly reduced as compared with wild-type conditions. These results further confirm the occurrence of a strong functional relationship between GRK2 cellular levels and signaling to MAPK cascades. Consistent with our data, decreased GRK2 cellular levels such as those present in heterozygous GRK2+/− mice leads to enhanced chemokine-mediated MAPK cascade activation.28 On the contrary, recent reports indicate that increased GRK2 cellular levels decrease β-adrenergic activation of the ERK/MAPK cascade (38, 39) or chemokine receptor-mediated MAPK stimulation.3

The existence of different pathways for the modulation of GRK2 stability may help understand its altered cellular levels in different pathologies and put forward new potential feedback mechanisms for regulating GPCR signaling. Such control mechanisms might be particularly relevant in pathological conditions characterized both by increased GPCR-mediated stimulation of Src and MAPK cascades and by altered GRK2 cellular levels, such as in heart failure, cardiac hypertrophy or hypertension (increased GRK2 levels), or chronic inflammation (decreased kinase levels) (20). It should be stressed, however, that phosphorylation-dependent modulation of GRK2 stability is not the only factor determining GRK2 cellular levels. A better knowledge of the signals governing GRK2 gene transcription is needed to better understand how this balance is altered in pathological situations characterized by GRK2 expression changes. It is also possible that such rapid processes of GRK2 turnover are not mainly intended to promote marked changes in steady-state GRK2 cellular levels but to preferentially target for degradation the active pool of GRK2 as a rapid feedback mechanism, as recently suggested for MAPK-dependent Ste11 degradation (35). Finally, in the light of recent reports (40, 41) suggesting the possible involvement of GRK2 and β-arrestin in receptor tyrosine kinase regulation and signaling, our finding of Src and MAPK phosphorylation-dependent modulation of GRK2 proteolysis suggests new potential cross-talk mechanisms between signaling pathways that deserve to be explored in the future.

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