Degradation of the G Protein-coupled Receptor Kinase 2 by the Proteasome Pathway*

Petronila Penela‡, Ana Ruiz-Gómez‡, José G. Castaño§, and Federico Mayor, Jr.‡‡

From the ‡Departamento de Biología Molecular, Centro de Biología Molecular “Severo Ochoa,” Consejo Superior de Investigaciones Científicas—Universidad Autónoma de Madrid, Universidad Autónoma, E-28049 Madrid, Spain and the §Departamento de Bioquímica e Instituto de Investigaciones Biomédicas, Facultad de Medicina, Universidad Autónoma, E-28029 Madrid, Spain

GRK2 is a ubiquitous member of the G protein-coupled receptor kinase (GRK) family and has been shown to play a key role in determining the desensitization and resensitization patterns of a variety of G protein-coupled receptors. In this report, we show that GRK2 is actively degraded by the proteasome proteolytic pathway, unveiling a new mechanism for the rapid regulation of its expression levels. Interestingly, activation of β2-adrenergic receptors (β2AR) markedly increases GRK2 ubiquitination and degradation through the proteasome pathway. In addition, blocking GRK2 degradation notably alters β2AR signaling and internalization, consistent with a relevant physiological role for GRK2 proteasomal degradation. Activity-dependent modulation of GRK2 cellular levels emerges as an important mechanism for modulating the cellular response to agonists acting through G protein-coupled receptors.

Changes in GRK2 expression may therefore alter the efficacy of GPCR signal transduction systems. In this regard, GRK2 expression levels have been reported to increase in human heart failure (10), hypertension (11) or upon lymphocyte activation (3), or to decrease rapidly in several rat tissues during the perinatal period.2 Despite recent advances in the understanding of the mechanisms regulating the activity and subcellular distribution of GRK2 (1, 2, 6, 12, 13), very little is known about the mechanisms that regulate the kinase cellular component. In this study, we sought to characterize the degradation process of GRK2 protein and its potential modulation. Our results clearly show that GRK2 is rapidly degraded by the proteasome pathway and that kinase degradation is modulated by activation of β2-adrenergic receptors (β2AR). Regulation of GRK2 stability may provide an important mechanism for modulating the cellular levels of this kinase and contribute to the regulation of desensitization/resensitization patterns of GPCR, as suggested by the fact that blockade of GRK2 degradation alters β2AR signaling as well as receptor internalization.

**EXPERIMENTAL PROCEDURES**

*This work was supported by Grant PM95-0033 from the Ministerio de Educación y Cultura, Grant AE 00213/95 from the Comunidad de Madrid, and Grant BMH4–98-3566 from the European Union (to F. M.) and grants from Comision Interministerial Ciencia y Tecnología and Fundación Ramón Areces (to J. G. C.). The Centro de Biología Molecular holds an institutional grant from the Fundación Ramón Areces. The Centro de Biología Molecular- Investigaciones Científicas-Universidad Autónoma de Madrid, Universidad Autónoma, E-28049 Madrid, Spain and the Centro de Biología Molecular holds a national grant from the Ministerio de Educación y Cultura, Grant AE 00213/95 from the Comunidad de Madrid, and Grant BMH4–98-3566 from the European Union (to F. M.).

† To whom correspondence should be addressed. Tel.: 34-91-397-48-65; Fax: 34-91-397-47-99; E-mail: fmayor@cbm.uam.es.

‡‡ The abbreviations used are: GPCR, G protein-coupled receptor; ALN, N-acetyl-Leu-Leu-norleucinal; β2AR, β2-adrenergic receptor; DMEM, Dulbecco’s modified Eagle’s medium; GRK, G protein-coupled receptor kinase; ISO, isoproteinem; MCP, multicatalytic proteinase complex; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; HA, hemagglutinin.

Agonist-promoted desensitization of G protein-coupled receptors (GPCR)1 involves rapid phosphorylation of the activated receptor by a family of specific G protein-coupled receptor kinases (GRK), followed by binding to the phosphorylated receptor of regulatory proteins termed arrestins, leading to uncoupling from G proteins (1, 2). GRK2 is the most ubiquitous member of this kinase family and has been shown to play a key regulatory physiological role in the heart and other tissues (3, 4). GRK2 may also play a role in facilitating the transient agonist-induced GPCR internalization allowing receptor dephosphorylation and recycling (5–7). Thus, GRK2 contributes to determine the rate and extent of both GPCR desensitization and resensitization. This fact, together with the recently suggested participation of GRKs and arrestins in the GPCR-mediated mitogen-activated protein kinase cascade (8) and the ability of GRK2 to phosphorylate tubulin (9), further stresses the relevance of GRK2 in GPCR signaling.

1 The abbreviations used are: GPCR, G protein-coupled receptor; ALN, N-acetyl-Leu-Leu-norleucinal; β2AR, β2-adrenergic receptor; DMEM, Dulbecco’s modified Eagle’s medium; GRK, G protein-coupled receptor kinase; ISO, isoproteinem; MCP, multicatalytic proteinase complex; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; HA, hemagglutinin.

2 P. Penela and F. Mayor, Jr., manuscript in preparation.

3 A. Ruiz-Gómez, unpublished data.

Vol. 273, No. 52, Issue of December 25, pp. 35238–35244, 1998
Printed in U.S.A.
GRK2 Degradation by the Proteasome Pathway

RESULTS

GRK2 Is Degraded by the Proteasome in Vivo and in Vitro—
The GRK2 degradation rate was measured by pulse and chase analysis using HEK-293 cells that stably express GRK2. In such cells, neither the subcellular localization pattern of the kinase nor the ratio between subcellular compartments was altered when compared with endogenous GRK2 (14). A rapid decay in the \(^{35}\)S-labeled GRK2 immunoprecipitated by a specific kinase antibody is shown in Fig. 1A, indicating that GRK2 is a short-lived protein with a half-life estimated at \(-60\) min. A similar value was obtained for the endogenous GRK2 in identical experiments performed in Jurkat cells (data not shown), ruling out the possibility that overexpression might alter the normal rate of kinase proteolysis. To ascertain which mechanism was responsible for the rapid GRK2 degradation, pulse and chase experiments were performed with specific inhibitors of different proteolytic pathways (Fig. 1B). Treatment with the cysteine protease inhibitor ALLN clearly prevents GRK2 degradation after 4 h of chase when compared with control conditions, at concentrations (50 \(\mu M\)) in which it inhibits the proteosome pathway but not at doses (1 \(\mu M\)) in which only nonproteosomal proteases are blocked (22), thus excluding the involvement of the latter. The presence of leupeptin, an inhib-
GRK2 Degradation by the Proteasome Pathway

Limited proteolysis of GRK2 by purified 20 S proteasome. A, recombinant GRK2 (80-kDa band) was incubated for the indicated times with or without (C120) purified 20 S proteasome (MCP) and resolved in SDS-PAGE followed by Coomassie Blue staining. An intermediate degradation product of 74 kDa is detected. MCP components are observed at the bottom of the gel. B, GRK2 was digested with MCP (control) or MCP plus different concentrations of lactacystin, resolved in 7% SDS-PAGE and analyzed as in panel A. C, GRK2 digested with (+MCP) or without MCP (C60) was resolved in 7% SDS-PAGE and immunoblotted with anti-GRK2 polyclonal antibodies Ab9 (dilution, 1:1000), AbFP2 (dilution, 1:600), and affinity-purified Ab1 (dilution, 1:20). Results are representative of two or three independent experiments.

To further address the role of the proteasome in regulating GRK2 stability, in vitro degradation experiments were conducted with recombinant GRK2 and purified 20 S proteasome from rat liver (18). The purified MCP preparations were judged homogeneous by SDS-PAGE analysis (Fig. 2A). Under the conditions tested, recombinant GRK2 (indicated as the 80-kDa protein in Fig. 2A) is indeed rapidly degraded, whereas GRK2 incubated for 2 h without MCP remains unaltered. To exclude the possibility that a minor contaminant protease present in the incubation could contribute to GRK2 proteolysis, we tested different MCP preparations for their ability to degrade GRK2, obtaining essentially the same results (data not shown). Moreover, different doses of the specific proteasome inhibitor lactacystin clearly prevent GRK2 cleavage as compared with control (Fig. 2B). Altogether, our data indicate that the kinase is a target for the proteasome, in vivo as well as in vitro. Interestingly, our in vitro GRK2 degradation experiments reveal the presence of a ~74-kDa band that appears as GRK2 decays (Fig. 2, A and C). This protein appears to represent an intermediate proteolytic product (also degraded by MCP following longer incubation times), whose formation is blunted in the presence of lactacystin (Fig. 2C). In a first attempt to determine whether the MCP initially digests the NH2- or the COOH-terminal region of GRK2 to yield the 74-kDa protein, we performed immunoblot analysis of GRK2 preparations incubated with or without MCP, using different GRK2 antibodies. Both intact GRK2 and the 74-kDa band are detected efficiently by the polyclonal antibody Ab9 raised against recombinant GRK2 and AbFP2, a polyclonal antibody against the COOH terminus (residues 436-689) (Fig. 2C). Moreover, antibody Ab1, which recognizes a more restricted epitope (GRK2 residues 648-655) that would be eliminated in an initial COOH-terminal cleavage, yields the same results (Fig. 2C, right panel), strongly suggesting that GRK2 is initially digested at its NH2 terminus.

The generation of the 74-kDa band as a result of GRK2 degradation in vitro raised the question of whether this cleavage product could also be observed in vivo. Fig. 3A indicates that the 74-kDa band is indeed immunodetected when measuring steady-state protein levels in cellular lysates from HEK-293 cells stably overexpressing GRK2 alone or with β2AR (Fig. 3A, lanes 4 and 5), whereas it is undetectable in untransfected cells (Fig. 3A, lane 1). Notably, the 74-kDa fragment seems to be more abundant in cells coexpressing GRK2 and a constitutively activated β2AR mutant (15) (lane 2 versus lane 3 in Fig. 3A). Moreover, we are able to immunoprecipitate the 74-kDa protein with a GRK2-specific antibody (Fig. 3B) but not with a preimmune serum (data not shown), when cells are metabolically labeled for periods longer than those used in pulse and chase experiments. These data indicate that the partial digestion of GRK2 by the proteasome takes place in situ and suggest that receptor activation might play some role in the process to render this product. We next determined the functional activity of the 74-kDa fragment by testing its ability to phosphorylate rhodopsin, a well known GRK2 substrate (12). For this purpose, kinase samples, undigested or digested with MCP, were purified by HPLC chromatography to remove MCP prior to the phosphorylation assay. The eluted fractions were resolved by SDS-PAGE and analyzed in Western blot (Fig. 3C). Most GRK2 was cleaved by MCP to yield the 74-kDa fragment. As can be seen in Fig. 3D, MCP-digested fractions (open circles) have less capacity to phosphorylate rhodopsin than undigested fractions (filled circles). Samples treated with MCP still display undigested GRK2 (~27%), which may account for the remaining activity. The decrease in activity (a 44% reduction) is lower, however, than that expected in such a case (a 73% reduction) indicating that the 74-kDa fragment can indeed phosphorylate rhodopsin, although with less efficiency than GRK2.

β2AR Activation Increases GRK2 Ubiquitination and Degradation—To explore whether receptor activation can modulate kinase turnover, we treated HEK-293 cells stably coexpressing GRK2 and β2AR with the β2-agonist isoproterenol (10 µM) or vehicle during different chase periods after 35S metabolic labeling (Fig. 4A). Under control conditions (Fig. 4A, −ISO), the levels of GRK2 decay essentially as described in cells expressing the kinase alone (see also Fig. 1A). Agonist stimulation (Fig. 4A, +ISO) clearly enhances the GRK2 degradation rate as compared with control (27% GRK2 remaining after 1 h of ISO treatment versus 54% in untreated cells; p < 0.05, data indicate the means ± S.E. of three to five independent experiments).
GRK2 Degradation by the Proteasome Pathway

GRK2 half-life thus decreases from 60 min (basal conditions) to ~30 min (stimulated turnover). The presence of lactacystin or ALLN completely abrogates GRK2 degradation in isoproterenol-treated cells (Fig. 4B), as they do in unstimulated cells. This ruled out the possibility that the increase in kinase processing triggered by receptor stimulation might arise from the activation of an additional proteolytic pathway other than proteasome-dependent degradation.

Most proteins targeted to the proteasome pathway undergo polyubiquitination (23, 24). To investigate the possible role of ubiquitination in basal and induced degradation of GRK2 in intact cells, we transiently transfected HEK-293 cells with different combinations of a tagged ubiquitin construct, GRK2, and β2-AR. In cells in which ubiquitin was overexpressed with the kinase and the receptor, a band smear corresponding to multi-ubiquitinated GRK2 (marked with a line in the right side of the upper panel in Fig. 4C) was clearly detected upon receptor stimulation with isoproterenol, whereas GRK2 ubiquitination was much lower in untreated cells (Fig. 4C, upper panel). Taken together, our data suggest that the extent of GRK2 ubiquitination is stimulation-dependent and probably accounts for the enhanced agonist-induced kinase degradation by the proteasome pathway.

Given that β2-AR occupancy by agonist promotes very rapid translocation of GRK2 to the plasma membrane and kinase activation (1, 2, 6, 13), we tested whether the activity of the kinase might also affect its turnover. To this end, HEK-293 cells stably expressing β2-AR and a dominant-negative GRK2 mutant (GRK2-K220R) (25) were metabolically labeled and chased in the presence (Fig. 4D, lanes I) or absence (Fig. 4D, lanes C) of isoproterenol. Agonist stimulation promotes no change in mutant kinase levels (Fig. 4D). More surprisingly, the amount of GRK2-K220R does not appear to decay even after 8 h of chase in the absence of agonist (Fig. 4D, upper panel), indicating that the mutant protein is far more stable than the wild type.

Blocking GRK2 Degradation Alters β2AR Signaling—The rapid and agonist-modulated degradation of GRK2 by the proteasome would provide a mechanism to regulate the cellular complement of GRK2 and, therefore, GPCR desensitization patterns. In this regard, we used HEK-293 cells stably expressing both β2-AR and GRK2 to test the effects of blocking GRK2 degradation with the specific proteasome inhibitor lactacystin on the subsequent cell response to the β-adrenergic agonist isoproterenol and on the β2-AR desensitization process. In control cells, agonist challenge promotes a 1.8 ± 0.1-fold increase in cAMP levels over basal values. Pretreatment with isoproterenol for 30 min induces the typical decrease in responsiveness to a subsequent agonist challenge (23 ± 7% decrease) (Fig. 5, left panel) and also a decrease in GRK2 levels (45 ± 9% of control), consistent with our previous data. Interestingly, inhibition of GRK2 degradation with lactacystin results in a clear increase in GRK2 protein levels both in control and isoproterenol-pretreated cells (Fig. 5, inset, lanes 2 and 4) and reduces β2-adrenergic receptor signaling by 33 ± 2 and 44 ± 1%, respectively (Fig. 5, left panel). Because cellular β2-AR levels remain unchanged with lactacystin treatments (see legend to Fig. 5), these data suggest that alteration of the kinase/receptor ratio clearly affects signal transduction. Other factors may also contribute to the reduction in β2-AR signaling in the presence of lactacystin, because regulatory subunits of cAMP-dependent protein kinase (26, 27) and other components of the transduc-
tion cascade could be degraded by the proteasome pathway. However, adenylyl cyclase activation by forskolin is not modified in the presence of lactacystin, and the same results were obtained in the presence of cAMP phosphodiesterase inhibitors (data not shown). Moreover, lactacystin treatment does not affect cAMP production by a transiently transfected truncated b2AR, which lacks phosphorylation sites for GRK2 but retains those for cAMP-dependent protein kinase and its ability to modulate adenylyl cyclase (Fig. 5, left panel), indicating that changes in GRK2 levels have a direct and crucial effect in the observed alteration in b2AR responsiveness.

Previous reports have provided evidence that GRK2 plays a facilitatory role in receptor internalization (5, 6, 28). Consistently, the blocking of kinase degradation with lactacystin markedly enhanced (up to 2.6-fold) receptor sequestration after a short term (2 min) challenge with isoproterenol or vehicle. These results are in agreement with our previous report (5) and further indicate that the impairment of the normal kinase turnover has important functional consequences on receptor desensitization and internalization processes.

**DISCUSSION**

Changes in the cellular complement of GRK2 can modulate the desensitization/resensitization processes of a variety of GPCR (7, 29) and may prove critical in determining the specificity of regulation of certain receptors (4, 7, 30). In this report, we show that GRK2 displays a rapid turnover through the ubiquitin-dependent proteasome pathway. This proteolytic pathway is responsible for the rapid degradation of an increasing number of cell cycle regulators and key signaling proteins (see Ref. 31 for a recent review). Changes in the stability of proteins as a consequence of dysregulated proteasome complex activity can lead to an impairment in the cellular functions mediated by these proteins (31). In this line, our data indicate that GRK2 degradation is modulated by GPCR activation and that blocking of kinase degradation strongly affects its role as regulator of GPCR function.

Proteins targeted to the proteasome pathway usually undergo polyubiquitination (23, 24), although this is not an absolute requirement (16, 32, 33). We show that GRK2 ubiquitination and proteolysis by the proteasome pathway is increased upon agonist activation of GPCR, although the kinase also exhibits weak ubiquitination under unstimulated conditions, suggesting that common mechanisms can regulate signal-dependent and basal GRK2 degradation in contrast to IkBa degradation (16). On the other hand, the ability of purified 20 S proteasome to digest GRK2 indicates that a direct interaction between the kinase and proteasome subunit(s) can occur. It is feasible that an undetermined structural motif in GRK2 con-
multimolecular complex (receptor/GRK2 and internalization (1, 5, 28). Data suggest the formation of a GRK2-mediated phosphorylation promotes arrestin binding to hypotheses can be considered to explain this observation. First, increase in GRK2 ubiquitination and degradation. Several hypo- positions can be considered to explain this observation. First, rise in GRK2 ubiquitination and degradation. Several hypoth-
oses can be considered to explain this observation. First, sses can be considered to explain this observation. First, GRK2-mediated phosphorylation promotes arrestin binding to the agonist-activated receptor, leading to receptor uncoupling and internalization (1, 5, 28). Data suggest the formation of a multimolecular complex (receptor/GRK2/β-arrestin) at specialized locations of the plasma membrane (5, 35–37) that will be internalized after recruitment of different proteins of the endocytic machinery. It is possible that interaction of GRK2 with molecules of this multimolecular complex plays a role in its signal-dependent degradation by locating the kinase in close proximity with the molecules responsible for its ubiquitination and/or allowing conformational changes that unmask kinase domains directly targeted for ubiquitination. In this regard, it is worth noting that the yeast G protein-coupled receptor Ste2p is ubiquitinated at the plasma membrane even if endocytosis is blocked (38, 39). Alternatively, the higher level of GRK2 ubiquitination due to agonist receptor activation might result from second messenger-mediated mechanisms or as a consequence of stimulated GRK2 activity leading to phosphorylation of unknown factors different from GPCR or to transient increases in kinase autophosphorylation, improving GRK2 modification by ubiquitination. Data obtained with the GRK2-K220R mutant are consistent with a strong relationship between the stability of GRK2 and its cellular function; the slow turnover of GRK2-K220R as compared with wild-type GRK2 is largely unaffected by receptor activation, the mutant is deficient in kinase activity, and it strongly impairs β-arrestin binding and the subsequent internalization (1, 28, 40).

Several recent reports suggest that both the extent and rate of receptor internalization as well as the pattern of desensitization/resensitization processes may vary depending on the cellular content of GRK2 and β-arrestin (7, 35, 35). Our data are consistent with these reports and, further, suggest that a tight regulation of GRK2 cellular levels by agonists is needed for normal receptor function and modulation. Indeed, the rise in GRK2 complement by blockade of its normal degradation pathway clearly enhanced the initial rate of receptor sequestration, according to the facilitatory role of GRK2 previously reported in this process (5, 28). More interestingly, receptor response to agonists is markedly affected in these conditions, indicating that in our cellular system a moderate increase of GRK2 over its steady-state levels is enough to evoke a strong change in GPCR signaling, putting forward the relevance of an adequate ratio between the receptor and its regulatory proteins. In this context, it is tempting to suggest that the increased down-regulation of kinase levels observed after sustained GPCR stimulation would help to preserve some degree of response to additional activation by agonists. In this regard, it is worth noting that GRK2 levels rapidly decrease in several tissues during the rat perinatal period,2 a physiological situation characterized by a dramatic and prolonged surge in plasma catecholamines. On the other hand, the impaired protein degradation by the proteasome pathway described in pathological conditions such as hypothyroidism (41) could explain the increased GRK2 levels detected by our laboratory in several

![Figure 5](image.png)

**Fig. 5.** Effect of blocking GRK2 degradation on β2-adrenergic receptor signaling and internalization. **Left panel,** stably transfected HEK-293 cells used in Fig. 4 or cells transiently transfected with GRK2 and a COOH-terminal truncated mouse β2AR were preincubated with isoproterenol or vehicle (control) for 30 min, resensitized, and finally challenged with isoproterenol for 10 min to both assess receptor-dependent adenyl cyclase activity and GRK2 levels by immunoblot (inset). Data are expressed as fold stimulation of cAMP levels over basal values in control cells (18 ± 4 pmol/mg protein). Fold stimulation in control conditions was taken as 100%. Treatments did not significantly alter total receptor levels in stable or transfected cells (28.6 ± 0.3 pmol/mg protein, respectively). Data are the means ± S.E. from two or three experiments performed in duplicate. **Right panel,** HEK-293 cells stably transfected with GRK2 and an epitope-tagged β2AR receptor were incubated with lactacystin or vehicle (control) for 3 h and receptor internalization in response to a 2-min challenge with the indicated concentrations of agonist analyzed by flow cytometry (see “Experimental Procedures”). Receptor internalization under control conditions was taken as 100%. Results are the means ± S.E. of three independent experiments performed in duplicate.
Acknowledgments—We thank Dirk Bohmann for tagged ubiquitin constructs, A. de Blasi for the Ab1 antibody, J. L. Benovic for the gift of GRK2 and GRK2-R220R cDNAs and the Ab9 antibody, and S. Cotechia for the constitutively active GRK2 and GRK2-K220R cDNAs and the Ab9 antibody, and S. Cotechia, C. Mark for editorial help, and Prof. F. Mayor for continuous encouragement.

REFERENCES
1. Bo¨hm, S. K., Grady, E. F. & Bunnett, N. W. (1997) Trends Pharmacol. Sci. 17, 416–421
2. Gros, R., Benovic, J. L., Tan, C. M. & Feldman, R. D. (1997) J. Clin. Invest. 99, 2087–2093
3. Ungerer, M., Bo¨hm, M., Elce, J. S., Erdmann, E. & Lohse, M. J. (1993) J. Biol. Chem. 268, 4625–4630
Degradation of the G Protein-coupled Receptor Kinase 2 by the Proteasome Pathway
Petronila Penela, Ana Ruiz-Gómez, José G. Castaño and Federico Mayor, Jr.

J. Biol. Chem. 1998, 273:35238-35244.
doi: 10.1074/jbc.273.52.35238

Access the most updated version of this article at http://www.jbc.org/content/273/52/35238

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 21 of which can be accessed free at http://www.jbc.org/content/273/52/35238.full.html#ref-list-1