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MAP kinase protects G protein-coupled receptor kinase 2 from proteasomal degradation ☆

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Abstract

The G protein-coupled receptor kinase 2 (GRK2) phosphorylates and shuts down signaling from 7-transmembrane receptors (7TMs). Although, receptor activity controls GRK2 expression levels, the underlying molecular mechanisms are poorly understood. We have previously shown that extracellular signal-regulated kinase (ERK1/2) activation increases GRK2 expression [J. Theilade, J. Lerche Hansen, S. Haunso, S.P. Sheikh, Extracellular signal-regulated kinases control expression of G protein-coupled receptor kinase 2 (GRK2), FEBS Lett. 518 (2002) 195–199]. In the present study, we found that ERK1/2 regulates GRK2 degradation rather than synthesis. ERK1/2 blockade using PD98059 decreased GRK2 cellular levels to 0.25-fold of control in Cos7 cells. This effect was due to enhanced degradation of the GRK2 protein, since proteasome blockade prevented down-regulation of GRK2 protein levels in the presence of PD98059. Further, ERK blockade had no effect on GRK2 synthesis as probed using a reporter construct carrying the GRK2 promoter upstream of the luciferase gene. We predict ERK1/2 mediated GRK2 protection could be a general phenomenon as proteasome inhibition increased GRK2 expression in two other cell lines, HEK293 and NIH3T3.

Keywords: G receptor kinase 2; ERK1/2 kinase; Degradation; Proteasome; PD98059; Lactacystin; 7TM; GPRC

The G protein-coupled receptor kinase 2 (GRK2) is an intracellular enzyme that phosphorylates 7-transmembrane receptors (7TMs). GRK2 is ubiquitously expressed and capable of phosphorylating a range of receptors [2]. GRK2 specifically recognizes and phosphorylates the activated form of receptors. Receptor phosphorylation promotes the binding of arrestin proteins that uncouple the G protein heterotrimer and support receptor internalization [3,4].

Both GRK2 activity and expression are controlled through GRK2s interactions with other intracellular molecules. GRK2 activity is regulated primarily by phosphorylation (for a review see [5,6]) exerted by several kinases including ERK1/2 that exerts a tonic inhibition on GRK2 activity through phosphorylation [7].

GRK2 expression is altered during physiological processes as well as in diseases. GRK2 expression is enhanced during prolonged 7TM receptor stimulation and vice versa treatment with 7TM receptor antagonists reduces GRK2 expression [8–11].

To elucidate the molecular mechanisms operating during PD98059 mediated GRK2 down-regulation, we

☆ *Abbreviations:* ALLN, *N*-acetyl-L-leucyl-L-leucyl-norleucinal; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; ERK1/2, early regulated signal kinase 1 and 2; GRK2, G protein-coupled receptor kinase 2′; kbp, kilobase pair; kDa, kilodaltons; MAP kinase, mitogen activated protein kinase; PBS, phosphate-buffered saline; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TBS, Tris-buffered buffered saline; 7TM, 7-transmembrane receptor a.k.a. G protein-coupled receptor; GPCR, G protein-coupled receptor.

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conducted a series of experiments. Here we show that ERK1/2 blockade decreases GRK2 levels by increasing GRK2 degradation. Furthermore, through inhibition of the proteasome, GRK2 protein was stabilized.

Methods

Reagents. Cell culture reagents and lipofectamine were from Lifetech. PD98059 was from New England Biolabs. ALLN and lactacystin, and all reagents for buffers were from Sigma. Plastic-ware for cell culture was purchased from CM-lab. Utensils and apparatus for Western blotting were from BioRad. Primary antibodies were from Upstate (anti-GRK2), New England Biolab (anti-ERK1/2), and Santa Cruz Biotech (polyclonal anti-GRK2, anti-cyclin D1, and anti-Rb). Secondary antibodies, enhanced chemoluminescence, and hyperfilms were from Amersham. The cDNA encoding GRK2 genes were kindly provided by Robert Lefkowitz, NC, USA. The GRK2 gene was subcloned into an adenoviral vector and propagated according to the manufacturer's manuals (Stratagene). The GRK2 promoter–reporter construct was a kind gift from Federico Mayor Jr. in Madrid, Spain, who designed the construct to encode a 1.6 kbp fragment of the GRK2 promoter inserted proximally to the luciferase reporter gene.

Cell culture and transfection. Cos7, HEK293, and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% v/v serum, 10 μg/ml non-essential amino acids, and 0.1 μg/ml gentamicin. Cells were treated with 10 μM PD98059 for the indicated time periods. ALLN (50 μM) was added 75 min prior to PD98059. Lactacystin (10 μM) was applied in the majority of experiments. In some experiments, the concentrations of the proteasome inhibitors were altered as indicated in the figures. Transient transfections with GRK2 were achieved using an E1-deficient type 5 adenovirus encoding the GRK2 gene. Expression of virus was confirmed using an immunoflourescence microscope, as the adenovirus carried a GFP gene in addition to the GRK2 wt.

For promoter analyses, the cells were transfected with lipofectamine essentially according to the protocol supplied by the manufacturer. Two days after transfection, media were changed to DMEM with 0.1% v/v serum, 10 μg/ml non-essential amino acids, and 0.1 μg/ml gentamicin. Subsequently, the specific drugs were added as indicated in the figures. All cell lysates were prepared as described below. Cells were washed twice in ice-cold PBS and lysed in a buffer containing 1% v/v Triton X-100, 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. After centrifugation (12,000*g*, 10 min, 4 °C), protein concentrations were assayed using the detergent insensitive assay provided by BioRad.

Analyses of endogenous GRK2 levels presupposed immunoprecipitation of 40 μg whereas analyses of overexpressed GRK2 were done by directly loading 20 μg of crude lysates onto 7.5% SDS–PAGE (polyacrylamide gel electrophoresis) gels. For immunoprecipitation cellular extracts were mixed with 1 μl of GRK2-specific antibodies for 2–4 h at 4 °C followed by incubation with 30 μl protein A–Sepharose beads for 2 h at 4 °C. Immunoprecipitates were washed trice with ice-cold lysis buffer before addition of 30 μl of 2× Laemmli buffer, boiling for 2 min, and loading of 25 μl sample for separation on 7.5% SDS–PAGE. Subsequent transfer to 0.22 μM PVDF membranes (150 mA/gel for 1.5 h) allowed immunodetection of proteins. Western blots were incubated in 5% w/v dried milk in TBST (TBS with 0.2% v/v Tween 20) for 15 min followed by incubation with primary antibodies for 45 min at RT (anti-GRK2 was diluted 1:2500 whereas all other primary antibodies were used 1:1000). Subsequently, the blots were washed trice in TBST and incubated with secondary horseradish peroxidase-conjugated antibodies (1:5000) for 1 h at RT. After three additional washes in TBST, the blots were developed using ECL reagent and exposed to hyperfilms. Densitometry was performed using laser densitometric analysis and the NIH Image 1.62 program. Reblotting with was possible after inactivation of the antibody-conjugated HRPO enzymes with NaN_3 (0.02% w/v) and subsequent application of antibodies with a different clonality.

Results

Since ERK activation increases GRK2 protein levels, we speculated that ERK blockade would decrease GRK2 levels. In Cos7 cells, the ERK blocker, PD98059, inhibited endogenous GRK2 expression within a 6 h incubation period while at 2 h no effect was observed (Fig. 1A).

Two main explanations for this result are possible since ERK1/2 could either stimulate GRK2 synthesis or prevent its degradation. To test if ERK blockade using PD98059 would have a direct effect on the activity of the GRK2 promoter, we transiently expressed a reporter–gene construct (containing a 1.6 kbp fragment of the GRK2 promoter upstream from the luciferase gene) in Cos7 cells [12]. We treated transfected cells with PD98059 for either 6 or 16 h and did not observe any reduction in the expression of the reporter gene expression (Fig. 1B). Similar results were obtained with a shorter promoter construct (0.3 kbp [12]) (data not shown). However, the GRK2 promoter could in fact react to stimuli since having 10% serum in the media induced luciferase gene expression (Fig. 1B). To examine if PD98059 possesses unspecific effects, we demonstrated

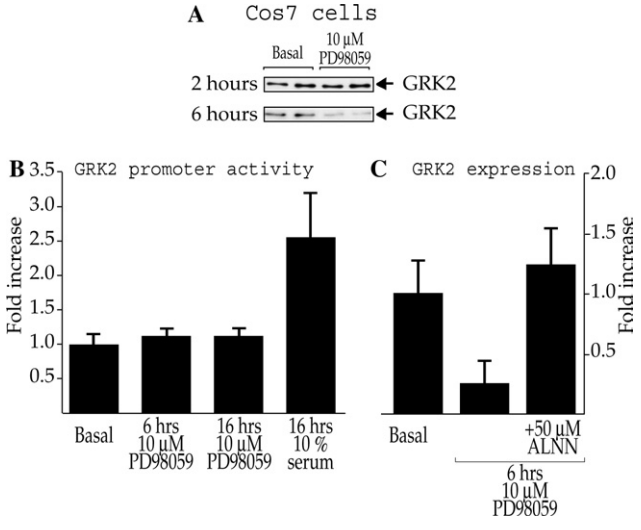

Fig. 1. (A) Blots represent duplicate samples. Treatment with 10 μM PD98059 decreased GRK2 expression during a 6 h period. Lysates from Cos7 cells were immunoprecipitated to visualize GRK2. Blots are representative of two to three experiments performed in duplicate. (B) The activity of the GRK2 promoter was assayed. A cDNA construct encoded a 1.6 kbp fragment of the promoter fused to the luciferase reporter gene. The histograms represent three independent experiments performed in duplicate. (C) Inhibition of proteasomal protein degradation prevented GRK2 down-regulation during PD98059 treatment. Histograms represent three experiments performed in duplicate.