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Review
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Atypical Actions of G Protein-Coupled Receptor Kinases

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Abstract

G protein-coupled receptor kinases (GRKs) and β-arrestins have been known as regulators of G protein-coupled receptors. However, it has been recently reported that GRKs and β-arrestins mediate receptor-mediated cellular responses in a G protein-independent manner. In this scheme, GRKs work as a mediator or a scaffold protein. Among 7 members of the GRK family (GRK1-GRK7), GRK2 is the most extensively studied in vitro and in vivo. GRK2 is involved in cellular migration, insulin signaling, and cardiovascular disease. GRK6 in concert with β-arrestin 2 mediates chemoattractant-stimulated chemotaxis of T and B lymphocytes. GRK5 shuttles between the cytosol and nucleus, and regulates the activities of transcription factors. GRK3 and GRK4 do not seem to have striking effects on cellular responses other than receptor regulation. GRK1 and GRK7 play specific roles in regulation of rhodopsin function. In this review, these newly discovered functions of GRKs are briefly described.

Key Words: G protein-coupled receptor kinase, Transcription factor, Desensitization, G protein-independent signaling

INTRODUCTION

Various stimuli from the environment are received by the cells and transmitted through cellular signaling machinery. G protein-coupled receptors (GPCRs) expressing on the cell surface are major players that receive the extracellular signals, and transmit them to intracellular signaling molecules. When agonists bind to GPCRs, the agonist-bound receptor activates G protein, and increases or decreases the catalytic activities of target molecules such as adenylyl cyclase and phospholipase C (Premont and Gainetdinov, 2007). To avoid overstimulation by GPCRs, the function of the agonist-bound GPCRs is downregulated by GPCR kinases (GRKs) and β-arrestins. The agonist-bound GPCRs are first phosphorylated by GRKs, which specifically recognize the active conformations of GPCRs. Then, the phosphorylated receptors are targeted by β-arrestins, which inhibit the interaction of GPCRs with G proteins.

The GRK family is classified into 3 subfamilies: GRK1, GRK2, and GRK4. The members of the GRK1 subfamily are GRK1 and GRK7. The GRK2 subfamily consists of GRK2 and GRK3, and the GRK4 subfamily includes GRK4, GRK5, and GRK6 (Willets *et al.*, 2003). Domain of GRK is divided into three parts: central part encodes kinase domain, and amino and carboxyl parts bind regulatory molecules (Fig. 1). GRK1 and GRK7 are selectively expressed in retina to regulate light response. GRK2, GRK5, and GRK6 are expressed throughout the body. GRKs have been recently reported to be involved in cellular signaling that is independent of receptor phosphorylation (Penela *et al.*, 2010a). GRK binds to and phosphorylates various molecules other than GPCRs. Among them, the function of GRK2 is most extensively studied, especially in the cardiovascular diseases (Belmonte and Blaxall, 2011). The function of GRK2 in the heart is particularly interesting, as GRK2 plays an essential role in progression of cardiac diseases such as heart failure and myocardial infarction. GRK5 has several functions other than the ability to phosphorylate GPCRs. GRK5 regulates the activities of nuclear factors κB (NF-κB) and histone deacetylase (HDAC) (Martini *et al.*, 2008; Sorriento *et al.*, 2008). GRK5 is also proposed to be involved in central neurological diseases such as Alzheimer's disease and Parkinson's disease (Chen and Feany, 2005; Cheng *et al.*, 2010). GRK2 and GRK6 participate in cell migration (Fong *et al.*, 2002; Penela *et al.*, 2008). As GRK1, GRK4, and GRK7 are expressed in specialized cells, atypical functions of these GRKs are fairly limited (Premont and Gainetdinov, 2007).

It has already been reported that GRK2 phosphorylates non-receptor substrates including tubulin, phosducin, ribosomal protein P2, synuclein, the inhibitory γ-subunit of cGMP PDE, and the β-subunit of the epithelial Na^+ channel (Penela *et al.*, 2003). Phosphorylation of these molecules suggests that GRK2 mediates the signals of GPCRs independent of G

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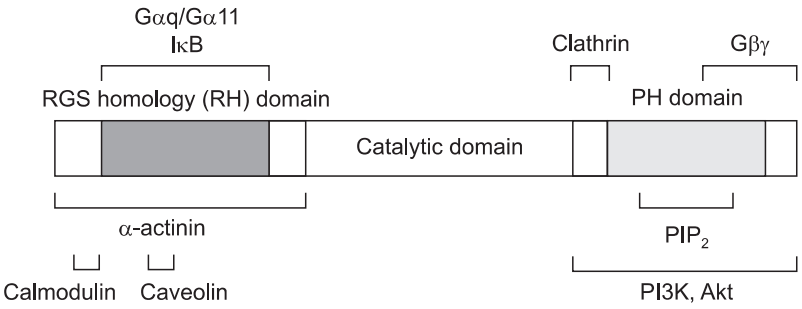


Fig. 1. Structure of GRK. Domain of GRK is divided into 3 parts. The catalytic domain is located at the central portion, and is flanked by 2 regulatory domains. The amino terminal domain has a RH domain, and the carboxyl terminal domain has a PH domain. Various molecules bind to amino or carboxyl terminal domains. PIP2: phosphatidylinositol-4,5-diphosphate, PI3K: phosphatidylinositol-3 kinase.

protein activation and desensitization. In the following section, I will briefly summarize GRK substrates other than GPCRs, and GRK-mediated cellular signaling that is independent of receptor desensitization.

GRK-MEDIATED CELL MIGRATION

Cells migrate to a particular site according to a gradient of chemoattractants. Chemotaxis is a complex process that starts with recognition of the ligands and transferring the signal to mechanical machinery. GRK6 was found to be involved in lymphocyte chemotaxis (Fong *et al.*, 2002). In splenocyte membranes of GRK6-KO mice, stromal cell-derived factor-1 (CXCL12) stimulated GTPase activity to the same extent as that of wild type mice, indicating that the chemokine receptor-G protein coupling is normal in GRK6-KO mice. However, chemotaxis of splenocytes to CXCL12 was strikingly impaired. Thus, GRK6 is essential for chemotaxis by activating signaling molecule(s) downstream of G proteins. As chemotaxis of splenocytes from β-arrestin 2-KO mice was also impaired, GRK6 may mediate CXCL12-stimulated chemotaxis by promoting the recruitment of β-arrestin 2 to the chemokine receptors. The target molecules of GRK6 that mediate chemotaxis remain to be determined. In addition to GRK6, GRK2 was also reported to participate in cell migration (Penela *et al.*, 2008). The increased expression of GRK2 enhanced migration of epithelial cells toward fibronectin. GRK2-mediated enhancement of cell migration was required by autocrine or paracrine secretion of sphingosine-1-phosphate (S1P) and activation of the S1P receptor. The effects of GRK2 were dependent on the phosphorylating activity of GRK2, interaction of GRK-interactor 1 (GIT1), and activation of the Rac/PAK/MEK/ERK pathway. Thus, GRK2 integrates fibronectin (extracellular matrix) and S1P receptor activation into epithelial cell migration.

GIT1 AS GRK-INTERACTING PROTEIN

GIT1 was identified as a protein that interacts with GRK2 by yeast two hybrid screening, and was found to inhibit β2-adrenergic receptor (β2AR) agonist-stimulated internalization (Premont *et al.*, 1998). As GIT1 is ARF-GAP, and ARF is proposed to be involved in internalization of the receptors, GIT1 recruitment promoted by GRK2 may actively participate in the internalization pathway. The differential sensitivity of internalization to GIT1 suggests that the receptors internalize via multiple pathways (Claing *et al.*, 2000).

GIT interacts and forms a complex with PIX (PAK-interacting exchange factor) (Hoefen and Berk, 2006). PIX is the GDP/GTP exchanger of Cdc42/Rac that functions to regulate cellular shape (i.e., membrane ruffling and cell spread) (Frank and Hansen, 2008). Thus, GRK2 may mediate the signal from receptor stimulation to produce morphological changes in cells. Interestingly, co-immunoprecipitation assays revealed that other GRKs, such as GRK3, GRK5, and GRK6, can also interact with GIT1 (Premont *et al.*, 2004). GRKs may be directly involved in GPCR-induced changes of cellular shape through Cdc42/Rac activation.

EZRIN AND RADIXIN AS GRK SUBSTRATES

Ezrin is a member of the ezrin-radixin-moesin (ERM) family of proteins that cross-link cortical actin to the plasma membrane (Hughes and Fehon, 2007). Ezrin was phosphorylated by GRK2 at the regulatory site responsible for maintaining it in an active conformation (Cant and Pitcher, 2005). This site is also phosphorylated by Rho-kinase and protein kinase C, which are known to participate in cellular migration and cellular shape changes. As GRK2-mediated phosphorylation of ezrin was insensitive to inhibitors of Rho-kinase and protein kinase C, phosphorylation of ezrin by GRK2 is a new pathway that links GPCR activation to cytoskeletal reorganization. Another member of the ERM family, radixin, is also phosphorylated by GRK2 (Kahsai *et al.*, 2010). GRK2-mediated activation of radixin was reported to regulate membrane protrusion and motility of epithelial cells. As GRK2 interacts with GIT1, GRK2 may play a central role in GPCR-stimulated morphological changes of cells through the interaction with GIT1 and ERM family proteins.

ACTIVATION OF LRP5/6-MEDIATED SIGNALING BY GRK5 AND GRK6

Wnt signaling regulates organogenesis and tissue regeneration through frizzled receptors and low density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) (Cadigan and Liu, 2006). Frizzled receptors are 7 transmembrane receptors that have structures common to the GPCR family. In contrast to frizzled receptors, LRP5/6 receptors are single transmembrane receptors. Phosphorylation of Ser/Thr in the repeated motif of LRP6 is necessary for LRP6 activation. Wnt-bound LRP5/6 receptors inhibited GSK-3β, leading to stabilization of β-catenin. Stabilization of β-catenin then increases transcriptional activities of transcription factors LEF and TCF. Phosphorylation of Ser/Thr of the proline-rich PPPSP motif

in LRP5/6 is essential for the receptor activation. GRK5 and GRK6 were found to be involved in LRP5/6-mediated signaling (Chen *et al.*, 2009). The expression of wild type, but not kinase-dead GRK5 and GRK6, together with LRP6, strongly activated Wnt signaling. GRK5 and GRK6 phosphorylated Ser in the proline-rich motif of LRP6 that leads to the receptor activation. In zebrafish, knockdown of GRK5 exhibited a similar phenotype to knockdown of LRP6. Expression of GRK5 recovered the responses in which β-catenin level was decreased. These results suggest that GRK5 and GRK6 phosphorylate the single transmembrane receptor LRP6 and activate downstream signaling.

INHIBITION OF APOPTOSIS BY GRK5-MEDIATED PHOSPHORYLATION OF P53

Tumor suppressor p53 determines cell fate and induces cell cycle arrest or apoptosis when the cells are exposed to various stresses (Vazquez *et al.*, 2008). Therefore, the unregulated increase or decrease of p53 results in detrimental effects on the cells. The level of p53 is primarily regulated by phosphorylation and proteasome-mediated degradation. Currently, p53 is known to be phosphorylated by HIPK2, ATM, Rad3-related, CHK2, and DNA-dependent protein kinase (Puca *et al.*, 2010; Chen *et al.*, 2010). GRK5, but not GRK2 and GRK6, phosphorylated Thr at position 53 (Thr53) of p53 in vitro, and this phosphorylation promoted degradation of p53 through a proteasome-dependent pathway (Chen *et al.*, 2010). Knockdown of GRK5 inhibited DNA damage-induced apoptosis in osteosarcoma cells. In GRK5-KO mice, the expression of p53 was increased in various tissues, and whole body γ-irradiation-induced apoptosis was enhanced. Thus, GRK5 is a novel kinase which regulates the stability of p53 through phosphorylation.

INHIBITION OF NF-κB TRANSCRIPTIONAL ACTIVITY BY GRK5

Intracellular localization of signaling molecules, including GRKs, is an important factor for efficient signaling (Scott and Pawson, 2009). The GRK4 subfamily (GRK4, GRK5, and GRK6) and the GRK1 subfamily (GRK1 and GRK7), but not other GRKs have a functional nuclear localization sequence and a nuclear exporting sequence (Cant and Pitcher, 2005). In addition to its nuclear localization/exporting sequences, GRK5 physically interacts with NF-κB p105 (Parameswaran *et al.*, 2006). NF-κB p105 is a precursor of NF-κB and regulates cytosolic signal transduction, but lacks transcriptional activity. Lipopolysaccharide (LPS)-stimulated NF-κB signaling is regulated by GRK5 in macrophages. LPS binds to Toll-like receptor 4 and activates ERK through TPL2, a MEK kinase. LPS stimulation releases TPL2 from the NF-κB p105-TPL2 complex, and TPL2 then activates ERK1/2. NF-κB p105 is phosphorylated by IκB kinase and degraded by proteasomes following LPS stimulation. GRK5 bound to and phosphorylated NF-κB p105, leading to inhibition of IκB kinase-mediated phosphorylation and degradation. The NF-κB family transcription factors consist of homo- or hetrodimers of p50 (a product of p105), p52 (a product of p105), p65 (RelA), c-Rel, and RelB (Gordon *et al.*, 2011). NF-κB mediates the expression of various cytokines such as VEGF, IL-8 and bFGF. In addition to NF-κB p105, GRK5 was found to physically interact with IκB-α and inhibit NF-κB transcriptional activity through promotion of IκB-α nuclear translocation (Sorriento *et al.*, 2008). The amino terminal domain of GRK5 interacted with IκB-α, and the expression of the amino terminal domain inhibited cytokine production and protection of apoptosis caused by NF-κB. Thus, GRK5 has an ability to interact with NF-κB precursor and IκB-α, and regulate NF-κB-mediated inflammatory signaling.

GRK5 AS A HISTONE DEACETYLASE KINASE

GRK5 is classified as a GRK4 subfamily, and has a nuclear localization signal (Cant and Pitcher, 2005). Sequence comparison reveals that all members of GRK4 and GRK1 subfamilies have a nuclear localization signal. It suggests that each member of the GRK4 and GRK1 subfamilies mediates additional signal upon GPCR stimulation. Transgenic overexpression of GRK5 in cardiac myocytes results in excess hypertrophy and worsening of heart failure by pressure overload (Rockman *et al.*, 1996; Eckhart *et al.*, 2000). Mechanistic analysis revealed that GRK5 acts as histone deacetylase-5 (HDAC5) kinase, because it is associated with and phosphorylated HDAC5, a repressor of myocyte enhancer factor 2 (MEF2) (Martini *et al.*, 2008). GRK5 then promoted the export of HDAC5 to the cytosol, and increased MEF2-mediated gene transcription activity. The size of the heart of transgenic mice overexpressing GRK5 was increased by pressure overload. Kinase-dead GRK5 blocked constitutively active Gαq-induced MEF2 activity. Thus, GRK5 is proposed to be involved in hypertrophic responses. However, it has not been examined whether pressure overload-induced hypertrophic responses in the heart are inhibited in GRK5 knockout mice. As GRK5 works as a HDAC5 kinase, the degree of hypertrophy and severity of heart failure by pressure overload should be reduced in GRK5 knockout mice. It remains to be determined whether GRK deficiency affects pressure overload-induced cardiac hypertrophy.

ROLES OF GRK5 IN G PROTEIN-INDEPENDENT SIGNALING

It has been demonstrated that GPCRs activate not only G protein-dependent, but also G protein-independent signaling pathways (Whalen *et al.*, 2011). The G protein-independent pathway is primarily mediated by β-arrestins. This G protein-independent pathway is positively and negatively regulated by GRK5. For instance, β-blocker-stimulated biased signaling through the β1-adrenergic receptor required GRK5- or GRK6-mediated phosphorylation of the receptors (Noma *et al.*, 2007). GRK5 was also found to mediate negative regulation of G protein-independent signaling through the phosphorylation of β-arrestin. Stimulation of the 5-HT_4 receptor with 5-HT activates Gs-dependent and -independent signaling pathways (Barthet *et al.*, 2009). The 5-HT-stimulated Gs-independent signaling pathway of the 5-HT4 receptor was inhibited by GRK5. This inhibition was caused by phosphorylation of β-arrestin 1. This result suggests that GRK5-catalyzed phosphorylation of β-arrestin 1 is a mechanism to downregulate G protein-independent signaling.

ROLE OF GRK5 IN ALZHEIMER'S DISEASE AND PARKINSON'S DISEASE

Alzheimer's disease is a form of dementia characterized by a decline in cognitive function and alteration of personality. In Alzheimer's patients, the number of cholinergic neurons is decreased, and inhibitors against acetylcholine esterase, which degrades acetylcholine, are used clinically (Terry and Buccafusco, 2003; Craig *et al.*, 2011). GRK5 phosphorylated and desensitized M2 muscarinic receptor function in vitro and in vivo (Walker *et al.*, 2004). The M2 muscarinic receptor is located presynapse in the hippocampus, and regulates acetylcholine release. In GRK5 knockout mice, presynaptic autoregulation by the M2 muscarinic receptor was abolished, and it caused prolonged and persistent activation (Liu *et al.*, 2009). Furthermore, the combination of a M2 muscarinic receptor blocker and a cholinesterase inhibitor corrected β-amyloid accumulation (Cheng *et al.*, 2010). These results suggest that GRK5 may control progression of Alzheimer's disease in mice.

Parkinson's disease is a degenerative disorder of the central nervous system (Dawson and Dawson, 2003). It is caused by the loss of dopaminergic neurons in the substantia nigra. In late stage of Parkinson's disease, dementia occurs in many patients who will eventually die of the disease. The α-synuclein gene has been suggested as being associated with inherited Parkinson's disease. GRK2 and GRK5 phosphorylated Ser129 of α-synuclein and GRK5 were found to colocalize with α-synuclein in the brain of sporadic Parkinson's disease patients (Pronin *et al.*, 2000). In a Drosophila Parkinson's disease model, GRK5-mediated phosphorylation of α-synuclein enhanced neurotoxicity (Chen and Feany, 2005). As a genetic association of the GRK5 gene with Parkinson's disease was found, it was suggested that GRK5 plays an important role in the pathogenesis of Parkinson's disease (Arawaka *et al.*, 2006). However, another group did not find a significant association of the GRK5 gene with Parkinson's disease at allelic, genotypic or haplotypic levels (Tarantino *et al.*, 2010). Thus, GRK5 can phosphorylate α-synuclein, but the role of the phosphorylation remains to be established.

POLYMORPHISM OF GRK5

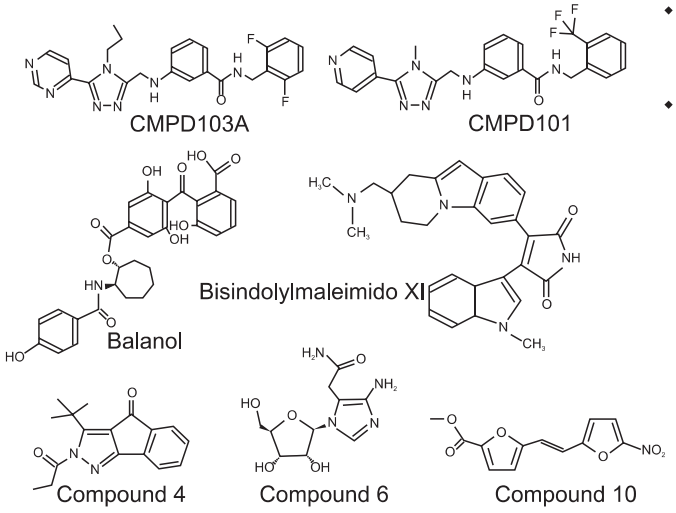
Genetic variation may determine the susceptibility to effects or side effects of medication (Wilke *et al.*, 2005). It has been reported that β1-AR and β2-AR have polymorphism in their genes, and some polymorphisms may be linked to cardiovascular diseases (Dorn, 2010). Among GRK2, GRK5, GRK6, β-arrestin 1, and β-arrestin 2, GRK5 has a genetic variation that changes amino acid Glu to Leu at position 41 (Leu41Gln) (Liggett *et al.*, 2008). In the report, Leu41Gln-GRK5 had the higher phosphorylating activity without agonist stimulation, and inhibited β-AR-induced cardiomyopathy in mice. The Leu41Gln mutation of GRK5 behaves as a gain-of-function mutant and then it functions as an endogenous genetic β-blocker. Leu41Gln polymorphism is 20-fold more common in African Americans than in Caucasians. It is suggested that this genetic variation explains the fact that treatment with β-blockers for heart failure patients is more effective for Caucasians than for African Americans. However, another group reported that Leu41Gln polymorphism of GRK5 is not associated with sensitivity to a β-AR blockade in humans (Kurnik *et al.*, 2009). They also showed that Leu41Gln variation does not contribute to the ethnic difference in sensitivity between African American and Caucasian populations to the β-blocker atenolol. Further study is necessary to establish the relationship between polymorphism of GRK5 and the sensitivity to β-blockers for the treatment of heart failure patients.

RGS HOMOLOGY DOMAIN OF GRK2

The regulator of G protein signaling (RGS) domains is encoded by -120 amino acids, and it selectively binds activated Gα and accelerates GTPase activity to turn off the signal (Zhang and Mende, 2011). Currently, >10 RGS proteins have been reported, and each RGS protein selectively interacts with Gαq or Gαi. The amino terminal portion of GRK2 and GRK3 encoded the RGS homology (RH) domain, which selectively binds Gαq/11 but not Gαs, Gαi, and Gα12/13 (Carman *et al.*, 1999). Comparable RH domains of GRK4, GRK5, and GRK6 did not bind Gαq and other Gα subunits. Binding of Gαq to GRKs caused phosphorylation-independent inhibition of Gq-coupled receptor stimulation. However, it has recently been reported that the RH domain of GRK2 has another function in receptor regulation (Fernandez *et al.*, 2011). The H_2 histamine receptor is a Gs-coupled GPCR, and histamine stimulation of the H_2 receptor increases cAMP through Gs. GRK2 desensitizes the H_2 receptor by phosphorylation of the receptor. As the RH domain at the amino terminal portion binds Gαq and Gαi, the RH domain of GRK2 is believed not to affect desensitization of the H_2 receptor. However, expression of the RH domain was sufficient to desensitize the H_2 receptor (Fernandez *et al.*, 2011). GRK2 catalytic activity was required for internalization of the H_2 receptor. Thus, GRK2-mediated desensitization of the H_2 receptor is induced by phosphorylation-dependent and -independent mechanisms. The RH domain of GRK2 may participate in receptor regulation in a more complex way than previously thought. The carboxyl terminal domain of GRK2 binds Gβγ and inhibits translocation and activation of GRK2 by receptor stimulation (Premont and Gainetdinov, 2007). In addition to the carboxyl terminal domain, the amino terminal domain was found to bind Gβγ. The binding of Gβγ to the amino terminal domain increased the phosphorylating activity of GRK2 (Eichmann *et al.*, 2003). The amino terminal domain of GRK2 may encode additional regulatory functions which mediate receptor desensitization.

GRK2 can interact with and interfere with the activation of MEK (Jiménez-Sainz *et al.*, 2006). GRK2 directly phosphorylated p38 MAPK and decreased its ability to interact with MKK6, an upstream activator (MEK) of p38 MAPK. Enhanced expression of GRK2 decreased p38 MAPK-dependent phosphorylation of MEF2, MK2, and ATF2. GRK2-mediated inhibition of p38 MAPK was independent of receptor phosphorylation. However, the site of interaction is not yet identified.

The amino terminal domains of GRK5 and GRK6 are important for receptor phosphorylation (Pao *et al.*, 2009). The combination of mutations at 2 different sites in the amino terminal domain of GRK5 decreased its ability to phosphorylate receptor and soluble substrates as well as autophosphorylation of GRK5. These results suggest that amino terminal domains of GRKs participate in the phosphorylating activity, rather than having GTPase-activation functions.



◆ Peptide inhibitor ($KRX\text{-}683_{124}$)
myristyl-Gly-Leu-Leu-Arg-Arg*-His-Ser-Ile-NH_2
Arg*: D-Arg

◆ Amino acid sequence of GRK2
59
Ala-Lys-Phe-Glu-Arg-Leu-Gln-Thr-Val-Thr-Asn-Tyr
74
-Phe-Ieu-Thr-Ser-Glu
Glu is added to carboxyl terminus.

Fig. 2. Chemical structure and amino acid sequence of GRK inhibitors. Structures of various GRK inhibitors are presented. Details for each inhibitor are described in the text.

PHOSPHORYLATION OF INSULIN RECEPTOR SUBSTRATE 1 (IRS1) BY GRK2

It has been reported that GRK2 expression level is increased in heart failure and myocardial infarction, and the increased expression of GRK2 has deleterious effects on the heart (Cipolletta *et al.*, 2009; Ciaccareli *et al.*, 2011). The strategy of lowering GRK2 activity and expression will improve cardiac function and reduces the progression to heart failure (Ciaccareli *et al.*, 2011). Insulin receptor substrate-1 (IRS1) is a new target of GRK2, and GRK2-mediated phosphorylation of IRS-1 inhibits insulin signaling. These results suggest that GRK2 activity inhibits insulin signaling in the heart and possibly in other tissues, which also suggests a relationship between GRK2 and regulation of metabolism (Mayor *et al.*, 2011).

INHIBITION OF eNOS ACTIVATION BY GRK2

Nitric oxide (NO) is an important mediator which causes relaxation of smooth muscle in blood vessels (Kawashima and Yokoyama, 2004). NO is synthesized by NO synthase (NOS), consisting of three subtypes: endothelial NOS (eNOS, NOS3), neuronal NOS (nNOS, NOS1), and inducible NOS (iNOS, NOS2). It was reported that NO production is decreased in sinusoidal endothelial cells, leading to portal hypertension when the liver is injured (Liu *et al.*, 2005). eNOS is activated by the increase in Ca^{2+}, and is also activated by Akt-mediated phosphorylation. Akt physically interacts with GRK2, and this interaction inhibits the interaction of Akt with eNOS; thus, GRK2 can indirectly inhibit eNOS. In sinusoidal endothelial cells, the reduced phosphorylation of Akt by overexpression of GRK2 resulted in hypertension of the portal vein. As GRK2 expression is increased in cardiovascular diseases, GRK2 and eNOS interaction may be critical for maintaining vascular endothelium in general.

GRK2 IN THE CELL CYCLE

The cell cycle is tightly regulated by various receptors, including GPCRs. While examining the role of GRKs in the cell cycle, GRK2 was found to participate in progression of the cell cycle (Penela *et al.*, 2010b). GRK2 levels were found to be transiently decreased at transition from G2 to M phases. Various cyclin-dependent kinase (CDK) inhibitors revealed that CDK2 is selectively involved in downregulation of GRK2. CDK2-catalyzed phosphorylation of GRK2 promoted the binding of prolyl-isomerase Pin1 to GRK2. The binding of Pin1 accelerated the degradation of GRK2. Mutation of GRK2 at the phosphorylation site by CDK2 inhibited the degradation and delayed the progression of the cell cycle. Although the target molecules of GRK2 for cell cycle progression are not yet identified, GRK2 participates in more cellular functions than previously thought.

GRKS IN DROSOPHILA

Smoothened (Smo) signaling is essential for development in vertebrates and invertebrates. Smo is a member of the GPCR family, and the ligand for Smo is Hedgehog. In Drosophila, there are 2 GRK genes: GPRK1 and GPRK2 (Molnar *et al.*, 2007). GPRK1 regulates visual transduction molecules such as rhodopsin kinase, and GPRK2 modulates cAMP levels during oogenesis. It has been reported that GRK2 phosphorylates mammalian Smo, and β-arrestin promotes internalization of Smo via a clathrin-coated pit (Chen *et al.*, 2004). To examine the role of GRK-mediated phosphorylation in Smo signaling, the GPRK2 gene in Drosophila was knocked out by insertion of LacZ (Molnar *et al.*, 2007). Mutant flies exhibited similar to those of the diminishing Smo signaling. As phosphorylation of Smo by GPRK2 is required for Smo signaling, proper trafficking of Smo to intracellular compartments may be necessary for induction of Smo-Hedgehog signaling in Drosophila. Using mammalian cells, it was also reported, that GRK2-catalyzed phosphorylation mediates Smo signal-

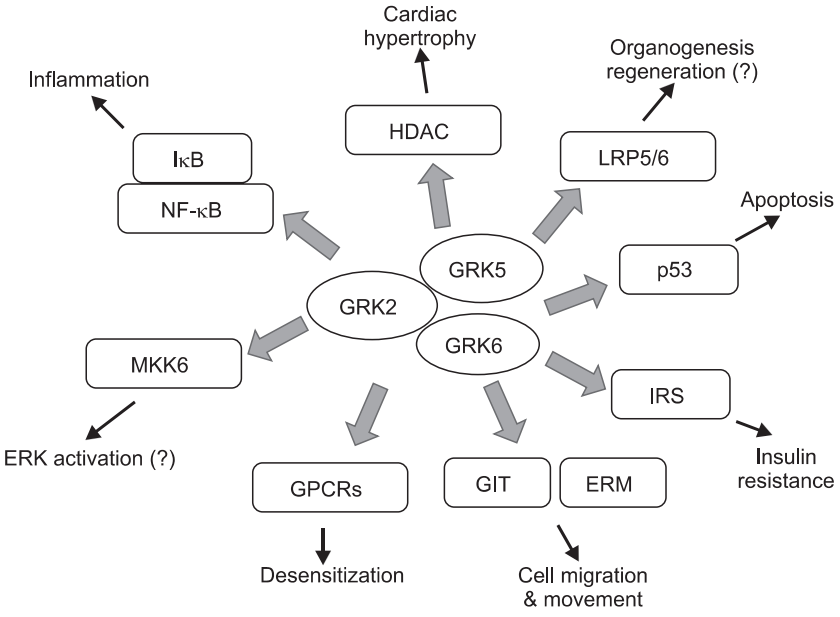


Fig. 3. Simplified scheme of GRK-mediated signaling pathways. GRK2, GRK5, and GRK6 interact with various signaling molecules, and increase or decrease their activities. The resultant changes in activities of these signaling molecules lead to responses in tissues. Thick arrows indicate the interaction, but do not mean that the interaction activates or inhibits signaling molecules. The details are described in the text.

ing by promoting β-arrestin binding to the phosphorylated Smo (Meloni *et al.*, 2006). Thus, GRK2 plays an important role in Hedgehog-Smo signaling in Drosophila and in mammalian cells. GRK2-catalyzed phosphorylation may mediate not only desensitization but also induction of β-arrestin-mediated cellular signaling in these cells.

INHIBITORS OF GRKS

When GRK subtype specific inhibitors are available, it is valuable to analyze the functions of each GRK in vitro and in vivo. Takeda compounds CMPD101 and CMPD103A inhibited GRKs, with a selectivity order of GRK2>GRK5>GRK1 (Thal *et al.*, 2011) (Fig. 2). Unfortunately, information on the affinities of these compounds for GRK3, GRK4, and GRK6 is not available. Crystal structure of GRK2 and CMPD103A complex revealed that CMPD103A binds GRK2 at the kinase active site (Thal *et al.*, 2011). As GRK2 plays an important role in progression of cardiovascular diseases such as heart failure and myocardial infarction (Rockman *et al.*, 2002; Brinks *et al.*, 2010), a selective GRK2 inhibitor should be useful for treatment of these diseases.

The chemical compound balanol was found to inhibit GRK2 (Setyawan *et al.*, 1999; Tesmer *et al.*, 2010). Because balanol can inhibit other kinases such as protein kinase A, protein kinase G, and protein kinase C, the contribution of these kinases to cellular responses must be tested when balanol is used as GRK inhibitor.

The carboxyl terminal portion of GRK2 (GRK2-ct: amino acids 542 to 685) including the pleckstrin homology (PH) domain is known as an inhibitor of GRK2. GRK2-ct inhibits GRK2 by trapping the Gβγ subunit, which is an activator of GRK2 (Koch *et al.*, 1999). However, GRK2-ct is not a selective inhibitor of GRK2, because the Gβγ subunit is involved in many cellular signaling pathways.

The peptide encoding the catalytic domain of GRK2, which interferes with the interaction with substrates, is expected to function as a selective inhibitor (Winstel *et al.*, 2005). Although data regarding the selectivity of this peptide is not available, this peptide may be used as a selective GRK2 inhibitor in cells by adding a penetrating sequence to cross the plasma membrane.

The peptide derived from GRK2 and GRK3 (KRX-683_{124}) improved glucose metabolism and had an antidiabetic effect (Anis *et al.*, 2004). However, the sequence of KRX-683_{124} is derived from that of the kinase-substrate interface of GRK2 and GRK3; KRX-683_{124} will also inhibit other GRKs.

The first intracellular loop of β2-AR showed selectivity toward GRK2 over protein kinase A and protein kinase C (Benovic *et al.*, 1990). However, this peptide also inhibited GRK3 and GRK5. Therefore, it is not a GRK subtype-selective inhibitor.

Ro32-043 (bisindolylmaleimide XI, known as a protein kinase C inhibitor) was found to be a less specific inhibitor of GRK5 (Aiyar *et al.*, 2000). Although Ro32-043 is a protein kinase C inhibitor, Ro32-043 had selectivity toward GRKs over protein kinase A when tested at micromolar concentrations. Because it also inhibited GRK2 and GRK3 with less potency compared to inhibition of GRK5, it is not considered as a selective inhibitor of GRKs.

A GRK2 inhibitor was developed by rational design (Iino *et al.*, 2002). Compounds-4, -6, and -10 in the report actually exhibited selectivity toward GRK2 over protein kinase A. However, the selectivity was not tested for other GRK subtypes. Thus, it is unknown whether these newly synthesized compounds are GRK2-selective inhibitors.

As each GRK has a different function in cells, the subtype selectivity is an essential factor to consider when using a GRK inhibitor for in vitro and in vivo experiments. Although several compounds were synthesized to develop GRK subtype-selective inhibitors, these efforts have been unsuccessful in producing subtype-selective inhibitors.

CONCLUSION

GRKs have been recognized as inhibitors of GPCR-stimulated signaling by phosphorylation. However, GRKs are also found to work as mediators of signaling. These GRK-mediated signaling pathways are independent of receptor phosphorylation (Fig. 3). Although GRKs can interact with and modify the activities of many proteins, the signaling pathway leading to GRK activation remains to be determined. As GRKs participate in many signaling pathway, unique targets of GRKs and new GRK-mediated signaling pathways will provide novel insights into the pathophysiology of various diseases.

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