Novel functions of GRK6 in macrophages by phosphorylating the non-GPCRs substrates

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G protein-coupled receptor kinases (GRKs) were initially identified as a family of serine/threonine protein kinases that regulate the activity of G protein-coupled receptors (GPCRs) [1]. GRKs greatly contribute to homeostasis by regulating the activity of more than 800 GPCRs encoded by the human genome [2]. When GPCRs are activated by the binding of their agonists, GRKs phosphorylate the intracellular domains of agonist-occupied receptors, and β-arrestins are recruited to the phosphorylation sites to inhibit further G protein-dependent signalling by interrupting receptor-G protein coupling (desensitization) [3]. Although GRKs consist of seven isoforms (GRK1–7) that are involved in G protein-dependent signalling, some differences have been observed in their expression patterns and functions [4, 5]. GRK2/3/5/6 are widely expressed throughout the body [6-8], in contrast, GRK1/7 and GRK4 are specifically expressed in the retina [9] and testis [10], respectively. GRK2 knockout mice die in the embryonic stage [11] and exhibit cardiac hypoplasia [12]. However, other GRKs are unessential for normal birth and development. Besides the inhibitory role of GRKs/β-arrestins in GPCR signalling, recent data indicated that GRKs can phosphorylate intracellular proteins other than GPCRs and regulate intracellular signalling [13]. For example, GRK2 is involved in inflammation, cell migration and insulin resistance by phosphorylating p38 mitogen-activated protein kinases [14], histone deacetylase 6 [15] and insulin receptor substrate 1 [16], respectively. GRK5 is involved in the inhibition of DNA-damage-induced apoptosis and the promotion of prostate cancer metastasis by phosphorylating p53 [17] or moesin [18], respectively. However, intracellular...
molecules that react with other GRKs, including GRK6, remain largely unknown.

Billions of cells die on a daily basis and are replaced as a result of normal tissue turnover. These dying cells expose phosphatidylserine on their surfaces as an ‘eat-me’ signal to phagocytes, such as macrophages and dendritic cells, which recognize this signal and swiftly engulf the dead cells. Because impaired engulfment by phagocytes is reported to cause autoimmune-like disease symptoms [19], the regulation of phagocytosis is very important for maintaining immune homeostasis. When phagocytes engulf dead cells, cytoskeletal rearrangement occurs via the activation of Rac1, which promotes the engulfment of apoptotic cells. However, the precise intracellular pathway(s) for apoptotic engulfment remains unknown. We recently demonstrated that GRK6 in macrophages associates with GIT1 to activate Rac1 and enhance the engulfment of apoptotic cells [20] in a kinase-dependent manner. We also showed that the engulfment pathway leading to Rac1 activation by the GRK6/GIT1 complex is independent of the well-known phagocytosis Dock180/Elmo/Rac1 and Glup1/Rac1 signalling pathways.

Because GRK6 kinase activity is crucial for the engulfment of apoptotic cells, we speculated that GRK6 promoted phagocytosis via phosphorylation of some unknown intracellular substrate(s). Thus, we employed a phosphoproteomic technique to identify new target proteins of GRK6 and revealed that GRK6 induces the phosphorylation of radixin and moesin, both of which belong to the ezrin/radixin/moesin family. These two proteins link actin filaments to the plasma membrane, thereby playing important roles in many cellular processes, including cell division, cytoskeletal remodelling and engulfment [21]. In fact, the phosphorylation of radixin and moesin in bone marrow-derived macrophages from GRK6-deficient mice decreased compared with that from wild-type mice, although GRK6 indirectly phosphorylated radixin and moesin. In support of this result, the phagocytic activity through GRK6 was attenuated by the knockdown of radixin and moesin in NIH3T3 cells. Therefore, these results suggest that phosphorylation of these proteins contributes to GRK6-mediated phagocytosis.

To elucidate the role of GRK6 in vivo, we focused on splenic macrophages because they highly express GRK6. The spleen is roughly divided into two regions, red pulp and white pulp, which remove senescent red blood cells and apoptotic B cells, respectively. In GRK6-deficient mice, more apoptotic cells were observed in the white pulp because of the defective engulfment by CD68-positive macrophages. Recent studies reported that the impaired removal of apoptotic B cells by CD68-positive splenic macrophages in the germinal centre leads to the development of autoimmune-like diseases, such as systemic lupus erythematosus (SLE) [22]. Thus, we determined whether serum levels of anti-dsDNA antibody, an important marker of SLE, increased in GRK6-deficient mice. Compared with wild-type mice, serum levels of anti-dsDNA antibody increased in GRK6-deficient mice. Furthermore, the deposition of immune complexes and the expansion of the mesangium in the kidneys, which are other important SLE-like symptoms observed in humans, were detected in GRK6-deficient mice. Thus, these results indicate that GRK6-deficient mice spontaneously develop an SLE-like autoimmune disease.

GRK6 is expressed not only by CD68-positive macrophages in the white pulp but also by F4/80-positive macrophages in the red pulp. As mentioned above, red pulp macrophages are responsible for removing senescent red blood cells. Thus, a defect in this process leads to iron deposition in the red pulp [23]. To determine the involvement of GRK6 in the engulfment of senescent red blood cells, we isolated F4/80-positive splenic macrophages from wild-type and GRK6-deficient mice and compared their abilities with phagocytise senescent red blood cells. We found that splenic macrophages derived from GRK6-deficient mice were...
significantly defective in the engulfment of senescent red blood cells compared with those from wild-type mice. The iron retained in senescent red blood cells was recycled through the swift engulfment by F4/80-positive splenic macrophages. In GRK6-deficient mice, iron deposition was observed in the red pulp. These results indicate that GRK6 mediates phagocytosis of senescent red blood cells in red pulp.

The results of the abovementioned study showed that GRK6 was abundantly expressed in several macrophages. Thus, we questioned whether GRK6 is involved in the immune responses elicited by macrophages. We assessed the contribution of GRK6 to NF-κB signalling, one of the most well-studied immune pathways in macrophages, and found that GRK6 phosphorylated IkBα at Ser32/Ser36 and enhanced TNF-α-induced inflammation in macrophages [24]. Phosphorylation of IkBα by GRK6 led to the degradation of IkBα and promoted NF-κB signalling. Moreover, GRK6 knockout in peritoneal macrophages significantly attenuated the transcription of inflammatory genes after TNF-α stimulation. Although IKK phosphorylates IkBα at Ser32/Ser36 and enhances TNF-α-induced inflammation [25], these results suggest that GRK6 has a function similar to that of IKK in NF-κB signalling.

GRKs phosphorylate intracellular non-GPCR substrates and regulate a variety of signalling pathways. However, it remains unknown how GRKs change their own activation states in the regulation of these pathways. The structural analysis of GRK6 [26] suggest that GRK6 assumes different conformations depending on its activation states. Therefore, we hypothesised that the structural changes of GRKs reflect their activation states. To monitor the conformational changes of GRK6 after TNF-α stimulation, we developed an intramolecular bioluminescence resonance energy transfer (BRET) probe with RLuc(h) (bioluminescent donor) and GFP² (fluorescent acceptor) fused to the N- and C-termini of GRK6, respectively. In the BRET technique [27, 28], the fluorescence intensity reflects the molecular distance between the bioluminescent donor and fluorescent acceptor. In particular, the fluorescence intensity of GFP² becomes stronger as the distance between RLuc(h) and GFP² is shortened in the presence of Deep Blue C, a substrate for RLuc(h). We overexpressed the RLuc(h)-GRK6-GFP² protein in HEK293 cells. When the cells were stimulated with TNF-α, the fluorescence intensity of GFP² gradually increased in a time-dependent manner. These results suggest that the conformation of GRK6 changes along with GRK6 activation by TNF-α. Thus, it will be beneficial to perform the BRET assay using macrophages and evaluate the activity of GRK6.

To summarize, we showed that GRK6 plays important roles in engulfment and inflammation by macrophages via phosphorylation of non-GPCR substrates. We demonstrated that (1) GRK6/GIT1 complex-activated Rac1 enhances engulfment independent of the two well-known phagocytosis-signalling pathways, (2) GRK6-deficient mice spontaneously exhibit an autoimmune-like disease probably due to the impairment of phagocytosis, (3) GRK6 phosphorylates IkBα at Ser32/Ser36 and promotes TNF-α-induced inflammation and (4) the structural change of GRK6 by TNF-α stimulation may reflect GRK6 activation. These results suggest that GRK6 has potential as a new therapeutic target to prevent autoimmune-like diseases and inflammatory disorders. Although inhibitors of GRK2 and GRK5 have been identified, such as paroxetine and amlexanox [29, 30], none have yet been discovered for other GRKs. Further, activators of all GRKs have yet to be identified. The GRK2 inhibitor paroxetine directly interacts with GRK2 and induces structural changes [29]. Therefore, it is expected that inhibitors or activators of GRK6 will also cause structural changes. Because our BRET assay system detects conformational changes of GRK6, this system may be useful to search for therapeutic compounds that modulate GRK6 activity.

Conflict of interest

All authors have no conflict of interest.

References


