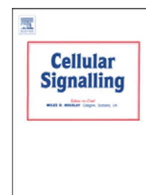
Cellular Signalling 25 (2013) 2831–2839

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig



G protein-coupled receptor kinase 2 (GRK2) is a Rho-activated scaffold protein for the ERK MAP kinase cascade

James D. Robinson, Julie A. Pitcher *

MRC Laboratory for Molecular Cell Biology, Research Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street, London WC1E 6BT, UK

ARTICLE INFO

Article history:
Received 11 March 2013
Received in revised form 30 August 2013
Accepted 31 August 2013
Available online 7 September 2013

Keywords:
G protein-coupled receptor kinase 2 (GRK2)
RhoA
Scaffold protein
ERK
Epidermal growth factor receptor (EGFR)

ABSTRACT

The G protein-coupled receptor kinases (GRKs) are best known for their role in phosphorylating and desensitising G protein-coupled receptors (GPCRs). The GRKs also regulate signalling downstream of other families of receptors and have a number of non-receptor substrates and binding partners. Here we identify $RhoA_{GTP}$ and Raf1 as novel binding partners of GRK2 and report a previously unsuspected function for this kinase. GRK2 is a RhoA effector that serves as a RhoA-activated scaffold protein for the ERK MAP kinase cascade. The ability of GRK2 to bind to Raf1, MEK1 and ERK2 is dependent on $RhoA_{GTP}$ binding to the catalytic domain of the kinase. Exogenous GRK2 has previously been shown to increase ERK activation downstream of the epidermal growth factor receptor (EGFR). Here we find that GRK2-mediated ERK activation downstream of the EGFR is Rho-dependent and that treatment with EGF promotes $RhoA_{GTP}$ binding and ERK scaffolding by GRK2. Depletion of GRK2 expression by RNAi reveals that GRK2 is required for EGF-induced, Rho- and ERK-dependent thymidine incorporation in vascular smooth muscle cells (VSMCs). We therefore hypothesise that Rho-dependent ERK MAPK scaffolding by GRK2 downstream of the EGFR may have an important role in the vasculature, where increased levels of both GRK2 and RhoA have been associated with hypertension.

© 2013 The Authors. Published by Elsevier Inc. Open access under CC BY license.

1. Introduction

The G protein-coupled receptor kinases (GRKs) are a family of seven serine/threonine protein kinases with important and varied roles in regulating cellular signalling [1,2]. They are separated into 3 subfamilies based on sequence homology. Both members of the GRK1 subfamily, GRKs 1 and 7, are expressed in the retina and GRK1 is additionally expressed in the pineal gland. The GRK2 subfamily, consisting of GRKs 2 and 3, and GRKs 5 and 6 of the GRK4 subfamily are widely expressed, while GRK4 is found at significant levels only in the testis, kidney and cerebellum [3]. All the GRK family members share a highly conserved central catalytic domain that is flanked by a more variable amino-terminal region and a poorly conserved, variable length carboxyl-terminal region [1,2].

The classical function of the GRKs is to phosphorylate and desensitise G protein-mediated signalling by G protein-coupled receptors (GPCRs), a family of approximately 800 cell-surface receptors with roles in regulating nearly all physiological processes. GRK2 is recruited to agonist occupied GPCRs *via* its carboxyl-terminal pleckstrin-homology (PH) domain which binds to activated Gβγ subunits in the presence of phosphatidylinositol-4,5-bisphosphate. Once bound to the activated GPCR, GRK2 is allosterically activated and subsequently phosphorylates the GPCR at serine and threonine residues usually within, either the third intracellular loop, or carboxyl-terminal tail. This triggers β-arrestin binding to the receptor, which prevents any further receptor-dependent activation of heterotrimeric G proteins. β-arrestins then initiate clathrin-coated pit-mediated receptor internalisation *via* recruitment of a number of proteins, including the clathrin adaptor AP2 [1,3].

Although best known for regulating GPCR signalling, GRK2 has also been implicated as a modulator of receptor tyrosine kinase (RTK) signalling [4]. In fact a number of parallels can be drawn between GPCR and RTK signalling, including coupling of some RTKs to heterotrimeric G proteins [5]. Akin to the well-documented agonist-dependent recruitment to GPCRs, GRK2 translocates to ligand-activated platelet-derived growth factor (PDGF) and EGFRs and phosphorylates their intracellular domains [6]. For the PDGF receptor, this results in reduced receptor activation reminiscent of GRK2-mediated desensitisation of GPCRs [7]. However, while recruitment of GRK2 to the EGFR is Gβγ-dependent [8], again reminiscent of its recruitment to GPCRs, this does not reduce receptor activation [9]. In fact GRK2 recruitment to the EGFR positively regulates EGF signalling, at least in terms of ERK activation [10,11].

Abbreviations: CNK1, connector enhancer of KSR-1; DGKθ, diacylglycerol kinase θ; EGF, epidermal growth factor; GIT, GRK-interacting protein; GPCR, G protein-coupled receptor; GRK, GPCR kinase; KSR-1, kinase suppressor of ras-1; MAPK, mitogen-activated protein kinase; PDEγ, phosphodiesterase-γ; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PKN, protein kinase N; ROCK, Rho-associated kinase; RTK, receptor tyrosine kinase; VSMC, vascular smooth muscle cell; RKIP, Raf kinase inhibitor protein.

* Corresponding author at: MRC Laboratory for Molecular Cell Biology, UCL, Gower Street, London WC1E 6BT, UK. Tel.: +44 2076797806.
E-mail address: julie.pitcher@ucl.ac.uk (J.A. Pitcher).

0898-6568 © 2013 The Authors. Published by Elsevier Inc. Open access under CC BY license.
http://dx.doi.org/10.1016/j.cellsig.2013.08.031

GRK2 can also regulate signalling at levels downstream of receptors, mediated by its phosphorylation of, and/or interactions with various non-receptor substrates and binding partners [12]. For example, the mitogen-activated protein kinase (MAPK) p38 is phosphorylated by GRK2 [13], preventing its activation by the upstream kinase MAPKK6. Phosphorylation of insulin receptor substrate-1 by GRK2 results in reduced insulin signalling [14], while GRK2-mediated phosphorylation of SMADs 2 and 3, reduces transforming growth factor-β signalling [15]. In contrast, M1 muscarinic receptor-dependent membrane ruffling is positively regulated by GRK2-mediated phosphorylation of ezrin [16]. Akt is one example of many non-receptor binding partners for GRK2. Binding to GRK2 inhibits the activation of Akt in sinusoidal endothelial cells, resulting in reduced nitric oxide production by Akt-activated endothelial nitric oxide synthase [17].

Given the importance of GPCRs in physiology, it is perhaps unsurprising that changes in the activity and/or expression levels of the GRKs are associated with a number of different disease states. GRK2 is particularly important in the pathology of cardiovascular diseases, including hypertension [18] and heart failure [19]. The true complexity of GRK2-mediated regulation of cellular signalling, for example at receptors other than GPCRs and at levels downstream of receptors is, however, only recently becoming appreciated and suggests that the role of GRK2 in these disease states may not be due simply to dysregulation of GPCR signalling. Here we identify Raf1 and Rho_{GTP} as novel GRK2 binding partners and demonstrate a new function for GRK2 as a scaffold protein for the ERK MAP kinase cascade downstream of the EGFR. Importantly, binding of GRK2 to Rho_{GTP} is required for the scaffolding function of GRK2. GRK2 thus represents a previously unappreciated Rho effector. GRK2 is required for Rho- and ERK-dependent thymidine incorporation downstream of the EGFR in vascular smooth muscle cells (VSMCs). We therefore hypothesise that this novel function of GRK2 as a Rho-activated ERK MAPK scaffold might have an important role in the vasculature, where increased levels of both GRK2 and RhoA have been associated with hypertension.

2. Materials and methods

2.1. Materials

Tissue culture dishes were obtained from Nunc, culture medium from Gibco BRL, plasticware from Falcon or Sterilin and other reagents from Sigma, unless otherwise stated. cDNAs: GRK5 (pRK5-GRK5) [20], GRK2 (pRK5-GRK2) [20], GRK3 (pcDNA1-GRK3) [20], GST-CT (pEGB-βARK-CT) [16], and GRK2-K220R (pBC12B1-βARK1-K220R) [16] were gifts from Prof. R. Lefkowitz. GRK6A, B, C (pBK(Δ)-GRK6A, B, C) [20] was from Dr. M. Tiberi. GRK2-NT (pEGFP-GRK2-(45-178)-GFP) [16] was from Dr. R. Sterne-Marr. Flag-GRK2 (pcDNA3-GRK2-Flag), and Flag-CAT (pcDNA3-GRK2-185-543-Flag) [21] were from Prof. Lan Ma. GST (pGEX6p1-EV), GST-RhoA-V14 (pGEX6p1-RhoA-V14), RhoA-WT (pRK5-MYC-RhoA-WT), RhoA-V14 (pRK5-MYC-RhoA-V14), RhoA-N19 (pRK5-MYC-RhoA-N19), RhoA-F25N (pRK5-MYC-RhoA-F25N) and RhoA-V14/F25N (pRK5-MYC-RhoA-V14/F25N) [22] were from Dr. S. Nurrish. Arf6-Q67L (pRK5-myc-Arf6-Q67L) [23], Rac-Q61L (pRK5-myc-Rac-Q61L) [24], Cdc42-Q61L (pRK5-myc-Cdc42-Q61L) [24] and Ras-V12 (pRK5-myc-Ras-G12V) [23] were from Prof. A. Hall. PTD-C3 (pGEX-KG-PTD-Myc-C3) [25] was from Prof. M. Olson. ERK2 (pEGFP-C1-ERK2) was from Dr. R. Seger. MEK1 (pRK5-myc-MEK-1) and Raf-1 (pEGFP-Raf-1) [26] were from Dr. P. Rodriguez-Viciana. Others were purchased from the Missouri S&T cDNA Resource Center. Antibodies were sourced as follows: Anti-phospho-ERK (9401) from Cell Signaling Technology. Anti-glutathione-s-transferase (GST) (27457701) from Amersham. Anti-green fluorescent protein (GFP) (11814460001) and anti-HA (1867423) from Roche. Anti-ERK (M5670) and anti-Flag (F3165) from Sigma. Anti-Myc (05-724) and anti-GRK4–6 (05-466) from Millipore. Anti-GRK2 (SC-562), anti-MEK (SC-6250), anti-Raf (SC-277), anti-PI3K (SC-7177) and anti-Gq (SC-393) from Santa Cruz Biotechnology, Inc. C3 Rho inhibitor was produced as described by Sahai and Olson [25].

2.2. COS, human embryonic kidney (HEK-293) and β-arrestin K/O mouse embryonic fibroblast (MEF) cell culture and transfection

Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum (FCS) and penicillin (100 IU/ml)/streptomycin (100 ug/ml) and were maintained in a humidified incubator at 37 °C, with 5% CO_2. Cells were transiently transfected using FuGENE HD (Roche) according to manufacturer's instructions.

2.3. Co-immunoprecipitation

Cells in 9 cm dishes were transfected as indicated. 48 h later they were washed twice with cold tris buffered saline (TBS) on ice and lysed by addition of 1 ml cold GTPase lysis buffer (10% glycerol, 50 mM Tris–HCl pH 8, 150 mM NaCl, 1% TX-100, 2 mM EDTA, 40 μg/ml PMSF, 1 mM benzamidine). After 15 s sonification, lysates were cleared by centrifugation at 13,000 rpm for 5 min. Protein concentrations were determined and volumes of lysates containing 200 μg protein were incubated with 2 μg of the indicated antibody for 1 h at 4 °C on a rotating platform. Protein A/G sepharose beads (GE Healthcare) were washed in GTPase lysis buffer and 30 μl of a 50% suspension was added to each sample before incubation for a further 1 h rotating at 4 °C. Beads were washed 4 times in GTPase lysis buffer before adding 25 μl SDS reducing buffer (25 mM Tris–HCl pH 6.5, 10% glycerol, 8% SDS, 5% β-mercaptoethanol, containing Brilliant Blue G) to each sample ready for analysis by SDS-PAGE. For quantification of GRK2 fragment binding to RhoA-V14, bands were quantified using Quantity One and the amount of GRK2 co-immunoprecipitated per unit RhoA-V14 was plotted relative to full length GRK2. For immunoprecipitation from EGF treated cells (Figs. 4 and 5) the same protocol was used but cells were serum starved for 24 h prior to EGF treatment and harvested in Buffer B (50 mM Tris pH 7.2, 150 mM NaCl, 7.5 mM $MgCl_2$, 1% TX-100, 40 μg/ml PMSF, 1 mM benzamidine, 100 μM Na_3VO_4, 10 mM β-glycerol phosphate, 20 mM NaF, 10 mM NaPPi). Immunoprecipitations were performed as described above using Buffer B throughout.

2.4. Direct binding assay

Full length GRK2 or GRK2 catalytic domain was *in vitro* translated using the Promega quick-coupled transcription/translation system according to the manufacturer's instructions. 10 μl of the *in vitro* translation product was then incubated with 10 μg purified GST or GST-RhoA-V14 on glutathione sepharose beads (GE Healthcare) in 200 μl IVT buffer (50 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.5% NP40) for 1 h at 4 °C on a rotating platform. Beads were then washed 4 times in IVT buffer before adding 25 μl SDS reducing buffer to each sample ready for analysis by SDS-PAGE and autoradiography.

2.5. ERK activation assay

HEK-293 cells were transfected, serum starved and treated with EGF as indicated. Harvesting was achieved by washing the cells with cold PBS and adding 200 μl SDS reducing buffer directly to each well. 50 μl of each sample was run on an SDS-PAGE gel to blot for levels of phospho- and total ERK. Films were scanned using a GS800 densitometer and bands were quantified using Quantity One. After subtraction of background, ERK activation was calculated by dividing intensity values for phospho-ERK by total ERK for each sample.

2.6. VSMC isolation, culture and nucleofection

VSMCs were isolated from rat aortas by enzymatic dissociation as previously described [27]. Cells were maintained at 37 °C, with 5% CO_2

in humidified conditions and culture medium (DMEM, 2 mM glutamine, 10% FCS (Gibco), penicillin 100 IU/ml/streptomycin 100 ug/ml) was changed every 3 days. The indicated concentrations of anti-GRK2 (si)RNA (5′-GCAGGUACCUCCAGAUCUCtt-3′) (Applied Biosystems) or scrambled control were nucleofected using Amaxa Biosystems primary smooth muscle cell nucleofection kit and nucleofector according to the manufacturer's instructions.

2.7. Thymidine incorporation

Nucleofected VSMCs in 12-well plates were serum starved and treated in the presence of [^{3}H]-thymidine (PerkinElmer) as indicated. Cells were lysed in 1 ml 1% SDS and transferred to pyrex tubes. 2 ml PBS was used to wash out wells and transferred to tubes. After addition of 3 ml ice cold 15% TCA (VWR), the tubes were vortexed and incubated on ice for 5 min. Samples were then filtered through pre-wet Whatman filter paper before washing the filter paper with 20 ml ice cold 5% TCA. Dried filters were then transferred to scintillation vials with addition of 6 ml Ultima Gold scintillation fluid (PerkinElmer). Incorporated [^{3}H]-thymidine was counted for 4 min using a Packard Tri-Carb liquid scintillation analyzer.

3. Results

3.1. RhoA$_{GTP}$ is a novel binding partner for GRK2

We identified RhoA$_{GTP}$ as a novel binding partner for GRK2 by performing co-immunoprecipitation experiments in COS cells. Myc-tagged small GTPase constructs were expressed with or without GRK2 co-expression. GRK2 co-immunoprecipitated with RhoA-V14 (a mutant that mimics the active, GTP-bound form of RhoA) but not with RhoA-N19 (a mutant mimicking the inactive, GDP-bound form) (Fig. 1A). GRK2 also failed to interact with constitutively active mutants of Rac (Rac-Q61L) and Cdc42 (Cdc42-Q61L) of the Rho GTPase superfamily and constitutively active mutants of other small GTPases (Arf6-Q67L and Ras-V12) (Fig. 1A). Thus, GRK2 appears to interact specifically with active RhoA. Further co-immunoprecipitation experiments with HA-tagged constitutively active mutants of RhoA, RhoB and RhoC indicate that GRK2 binding is common to the RhoABC subfamily of Rho GTPases (Fig. 1B). Despite different cellular functions of RhoA, B and C, known Rho effectors, including PKN, commonly bind to all three Rho isoforms [28]. We also observe co-immunoprecipitation of GRK2 with RhoA-V14 in β-arrestin knockout MEFs (Fig. 1C), indicating that the interaction between these two proteins occurs independently of β-arrestins and is not cell type specific. RhoA-V14/F25N is a stabilized mutant of RhoA originally designed to aid in its purification. This mutant binds normally to most RhoA effectors, including PKN, mDia and Rho-associated kinase (ROCK) but is impaired in its binding to some effectors such as diacylglycerol kinase θ (DGKθ) [22]. GRK2 binds poorly to RhoA-V14/F25N (Fig. 1D) suggesting that GRK2 may belong to a 'DGKθ class' of Rho effectors. Rho binding by DGKθ is mediated by its C-terminal 152 amino acid accessory domain [22] however, the residues required for Rho binding have not been definitively mapped.

In order to identify which region of GRK2 is responsible for Rho$_{GTP}$ binding, we co-expressed tagged constructs of the amino-terminal domain, amino acids 45–178 (GFP-NT), catalytic domain 185–543 (CAT-Flag) and carboxyl-terminal domain 492–689 (GST-CT) of GRK2 with Myc-tagged RhoA-V14 in COS cells. CAT-Flag [21], GFP-NT [16] and GST-CT [16], have previously been shown to fold and function normally in cells (see Materials Section for full list of construct sources and references). As assessed by co-immunoprecipitation, the catalytic domain of GRK2 exhibits the strongest interaction with RhoA-V14 (Fig. 2A, top panel), confirming that GRK2 binds to Rho$_{GTP}$, at least in part, *via* its catalytic domain. Quantifying the amount of GRK2 co-immunoprecipitated per unit of RhoA-V14 immunoprecipitated reveals that the catalytic domain of GRK2 binds three times better than the full-length GRK2 construct (3.0 ± 0.4 fold, $P < 0.01$) suggesting that, *in vivo*, a conformational change in GRK2 may be required to expose the GRK2 catalytic domain

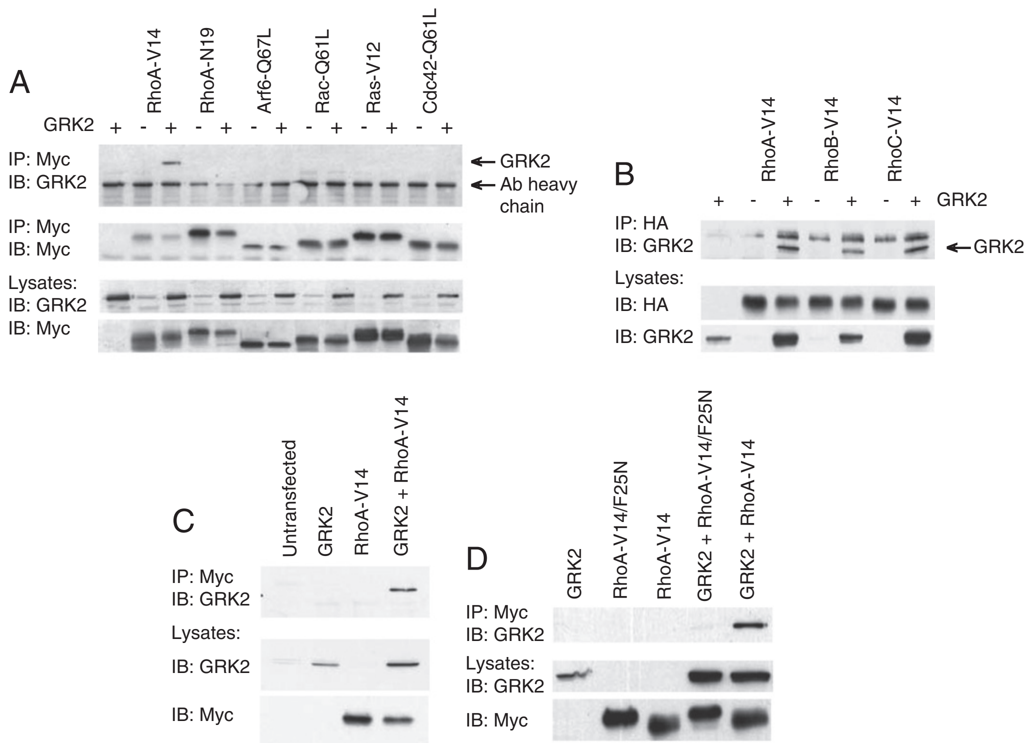


Fig. 1. GRK2 interacts specifically with active Rho. COS cells (A, B and D) or β-arrestin KO MEFs (C) were transfected as indicated. Protein expression was confirmed by Western blotting (lysates) with the indicated antibodies. Myc-tagged small GTPases were detected using an anti-Myc antibody in (A), (C) and (D) and HA-tagged small GTPases using an anti-HA antibody in (B). Myc- or HA-tagged small GTPases were immunoprecipitated (IP) and immunoprecipitates were probed (IB) for GRK2 content using an anti-GRK2 antibody. Western blots are representatives from 3 separate experiments.

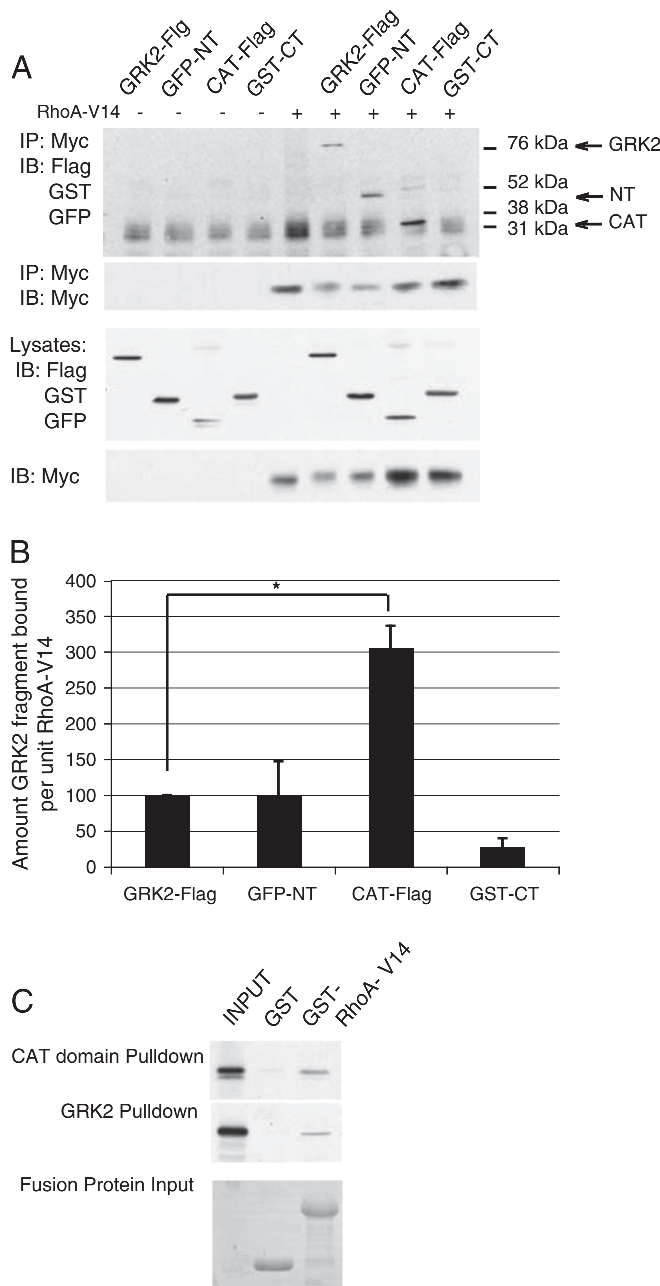


Fig. 2. GRK2 interacts directly with active RhoA, *via* its catalytic domain. (A) COS cells were transfected as indicated and protein expression was confirmed by Western blotting (lysates). Myc-tagged RhoA-V14 expression was confirmed using an anti-Myc antibody while expression of GRK2-Flag, GFP-NT, CAT-Flag and GST-CT was assessed by incubating the membrane with a mixture of anti-Flag, GFP and GST antibodies. Detected levels of the different constructs were similar under these conditions (see 'lysates'). Myc-tagged RhoA-V14 was immunoprecipitated (IP) from each sample and immunoprecipitates were probed (IB) for the presence of GRK2 constructs using the same antibody mixture. The Western blots shown are representative of 3 separate experiments. (B) Quantification of (A), the amount of GRK2 or GRK2 fragment co-immunoprecipitated per unit RhoA-V14 immunoprecipitated was determined. The amount of full length GRK2 co-immunoprecipitated per unit RhoA-V14 immunoprecipitated was arbitrarily set to 1.0 in each experiment, error bars represent standard deviation from the mean of 3 separate experiments. (C) GST or GST-RhoA-V14 was used to pull down *in-vitro* translated GRK2 or GRK2 catalytic domain. Samples were run on SDS-PAGE gels alongside 1 μl of *in vitro*-translated GRK2 or GRK2 catalytic domain input and gels were dried and exposed to film. The fusion protein inputs were checked by running equal amounts of GST and GST-RhoA-V14 on SDS-PAGE gels before coomassie staining. Western blots are representative of 3 separate experiments.

for full Rho_{GTP} binding (Fig. 2B). While the interaction of RhoA-V14 with the carboxyl terminus (CT) of GRK2 is poor, its interaction with the amino terminus (NT) of GRK2 is as strong as with the full-length kinase, indicating that the amino-terminal domain of GRK2 may also contribute to RhoA-V14 binding (Fig. 2A and B).

GRK2 is known to interact with Arf6 indirectly *via* GRK-interacting protein (GIT1) [29]. That GRK2 co-immunoprecipitates with RhoA-V14, but fails to do so with Arf6-Q67L (Fig. 1A), may indicate that the interaction between GRK2 and Rho_{GTP} is direct. To test this, we performed GST pull-down experiments with a GST-RhoA-V14 fusion protein or a GST negative control (Fig. 2C). The purified fusion proteins were incubated with *in vitro*-translated [^{35}S]-methionine-labelled GRK2 or the GRK2 catalytic domain (amino acids 185–543). *In vitro*-translated GRK2 and GRK2 catalytic domain both bound to GST-RhoA-V14 but not to GST (Fig. 2C). Taken together, these results indicate that GRK2 interacts directly with Rho_{GTP} *via* its catalytic domain, supporting a novel role for GRK2 as a Rho effector.

3.2. $RhoA_{GTP}$ binding to GRK2 promotes GRK2/Raf1, GRK2/MEK1 and GRK2/ERK2 complex formations

Co-expression of RhoA-V14 with GRK2 in COS cells or treatment of GRK2-transfected cells with the RhoABC inhibitor C3 had no effect on GRK2-mediated desensitisation of inositol trisphosphate production downstream of the angiotensin receptor, as assessed by measuring [^{3}H]-inositol incorporation into inositol phosphates in response to angiotensin II (ANG_{II}) (data not shown). Similarly, GRK2-mediated rhodopsin phosphorylation was unaffected by RhoA-V14 co-expression, as assessed by *in vitro* kinase assays using cell lysates expressing GRK2 with or without RhoA-V14 co-expression (data not shown). Since RhoA-V14 does not appear to effect the classical role of GRK2 in phosphorylating and desensitising GPCRs, we looked to see whether RhoA-V14 binding to GRK2 affects its ability to interact with any of its previously identified non-receptor binding partners. To do this, GRK2 was co-expressed in COS cells with four such binding partners; phosphoinositide 3-kinase (PI3Kγ); MEK1; Gq or GIT1 [2]. Under our experimental conditions GRK2 co-immunoprecipitated with PI3Kγ, GIT1 and Gq but not with MEK1 (Fig. 3A, B, C and D). Upon co-expression of RhoA-V14, the co-immunoprecipitation of GRK2 with PI3Kγ, GIT1 and Gq was equivalent to that seen in the absence of RhoA-V14 expression, indicating that RhoA does not affect GRK2 binding to these binding partners (Fig. 3A, B and C). In marked contrast, co-immunoprecipitation of GRK2 with MEK1 was strongly potentiated by co-expression of RhoA-V14 (Fig. 3D), suggesting that RhoA-V14 binding to GRK2 acts to promote GRK2 binding to MEK1.

ERK scaffold proteins bind to two or more of the components of the ERK MAPK cascade in order to promote their phosphorylation and hence regulate the strength, duration and/or subcellular localisation of ERK activation. This is important to regulate the specificity of cellular responses downstream of ERK. We therefore wanted to test whether GRK2 could also bind to the upstream MAPKKK Raf and/or the downstream MAPK ERK. As for MEK1, co-immunoprecipitation of GRK2 with Raf1 or ERK2 is potentiated by co-expression of RhoA-V14 (Fig. 3E and F). While MEK1 [30] and ERK [31] are previously characterised as binding partners for GRK2, the observed interaction with Raf1 has not previously been shown. Thus, RhoA-V14 expression promotes the interaction of GRK2 with all 3 components of the Raf/MEK/ERK MAP kinase cascade. These results suggest that GRK2 may have a novel function as a Rho-activated ERK MAPK scaffold protein.

That GRK2 binding to MEK1 is a direct consequence of $RhoA_{GTP}$ binding to GRK2 is indicated by the following observations. Firstly, GRK2 interacts specifically with the active mutant of RhoA, RhoA-V14, but not with the inactive mutant, RhoA-N19 (Fig. 1A). Consistent with this observation RhoA-V14, but not RhoA-N19, potentiates co-immunoprecipitation of GRK2 with MEK1, when overexpressed in COS cells (Fig. 4A). Furthermore, RhoA-V14/F25N, a mutant of RhoA-V14 that does not interact with GRK2 (Fig. 1D) but interacts normally with most other effectors, including ROCK [22], also fails to promote binding of GRK2 to MEK1 (Fig. 4B). Thus $RhoA_{GTP}$-dependent binding

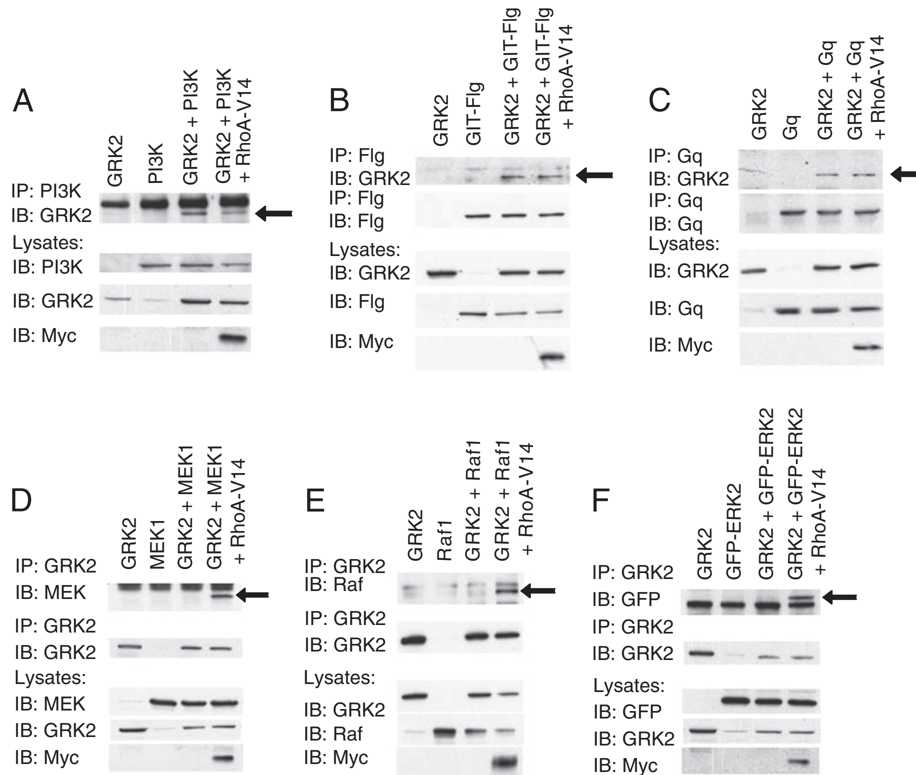


Fig. 3. $RhoA_{GTP}$ binding to GRK2 promotes GRK2 binding to Raf1, MEK1 and ERK2. COS cells were transfected as indicated and expression of proteins was confirmed by Western blotting (lysates). Expression of RhoA-V14 was confirmed by blotting with an anti-Myc antibody, GIT1-Flag with an anti-Flag antibody and GFP-ERK2 with an anti-GFP antibody. All other proteins were detected using the appropriate specific antibodies as detailed in the methods. Following immunoprecipitation (IP), the immunoprecipitates were probed (IB) with antibodies against the indicated proteins/epitopes. Western blots are representative of at least 3 separate experiments.

of GRK2 to MEK is most likely a direct consequence of $RhoA_{GTP}$ binding to GRK2 rather than an indirect consequence of RhoA signalling. Taken together, these results support a novel function for GRK2 as a Rho effector that binds Raf1, MEK1 and ERK2 in response to $RhoA_{GTP}$ binding in COS cells.

In order to test downstream of which receptors Rho-mediated Raf/MEK/ERK binding by GRK2 may occur, we next treated COS cells with a variety of agonists and looked to see if any affected the interaction between GRK2 and RhoA-V14. We found that the co-immunoprecipitation of GRK2 with RhoA-V14 is 2.0 $\pm$ 0.5 fold greater in cells treated with EGF, relative to untreated cells (Fig. 4C top panel, 4th and 5th lane and quantified in 4D). This is also the case for kinase dead GRK2 (GRK2-K220R), which interacts 2.5 $\pm$ 0.7 fold more strongly with RhoA-V14 in cells treated with EGF relative to untreated cells (Fig. 4C top panel, 6th and 7th lane and quantified in 4D). Thus, EGF treatment of COS cells promotes RhoA-V14/GRK2 complex formation, in a kinase-independent manner, suggesting that Rho-mediated ERK scaffolding by GRK2 might occur downstream of the EGFR.

3.3. GRK2 promotes ERK activation downstream of the EGFR by acting as a Rho-dependent scaffold protein

Overexpression of GRK2 in HEK-293 cells has previously been shown to potentiate ERK activation downstream of the EGFR [10,11]. Since agonist occupancy of the EGFR also activates Rho [32] and we have found that EGF treatment promotes the interaction between GRK2 and RhoA-V14 (Fig. 4C and 4D), we investigated whether Rho-activated ERK scaffolding by GRK2 may, at least in part, account for the GRK2-mediated increase in EGF-induced ERK activation that has been reported in these cells. Consistent with previously published results [10,11], we find that overexpression of GRK2 in HEK-293 cells results in a ~2-fold increase in EGF-induced ERK activation, as detected by Western blotting for phospho-ERK (Fig. 5A and B). Exogenous GRK2 does not however affect the overall time course of ERK activation; phospho-ERK levels peak at 2–5 min and return to basal levels after 10 min in both control and transfected cells (Fig. 5B). The GRK2-mediated increase in ERK activation in response to EGF in these cells is abolished by pretreatment with the RhoABC inhibitor C3 (Fig. 5C). Thus GRK2-dependent ERK activation downstream of the EGFR appears to be Rho-dependent.

If GRK2 acts as a Rho-activated ERK scaffold downstream of the EGFR, then we would expect to observe the association of GRK2 with endogenous activated P-ERK following EGF treatment. Indeed, immunoprecipitation of Flag-tagged wildtype or kinase dead (K220R) GRK2 from cells treated with EGF for 5 min reveals the presence of endogenous activated ERK within the immunoprecipitates (Fig. 5D). This result suggests that GRK2 functions as an ERK scaffold in a kinase-independent fashion. The amount of endogenous activated ERK that co-immunoprecipitates with overexpressed GRK2 in response to EGF treatment is increased by co-expression of RhoA-V14 (Fig. 5E), providing further evidence that ERK scaffolding by GRK2 is $RhoA_{GTP}$-dependent. Finally, we find that, in response to EGF treatment, we can detect an interaction between endogenous GRK2 and endogenous activated ERK (Fig. 5F), demonstrating that this function of GRK2 is not an artefact of its overexpression. Taken together, these results suggest that Rho-activated ERK scaffolding by GRK2 can function to increase EGF-induced ERK activation in HEK-293 cells.

3.4. GRK2 is required for EGF-induced proliferation of VSMCs

Several studies have linked increased levels of GRK2 with hypertension in both patients and animal models [18]. Transgenic mice with GRK2 specifically overexpressed in their VSMCs have increased blood pressure associated with vascular thickening and eventually die of heart failure [33]. Aberrant EGFR signalling [34], Rho activity and Rho expression levels [35] have also been linked to hypertension. We thus

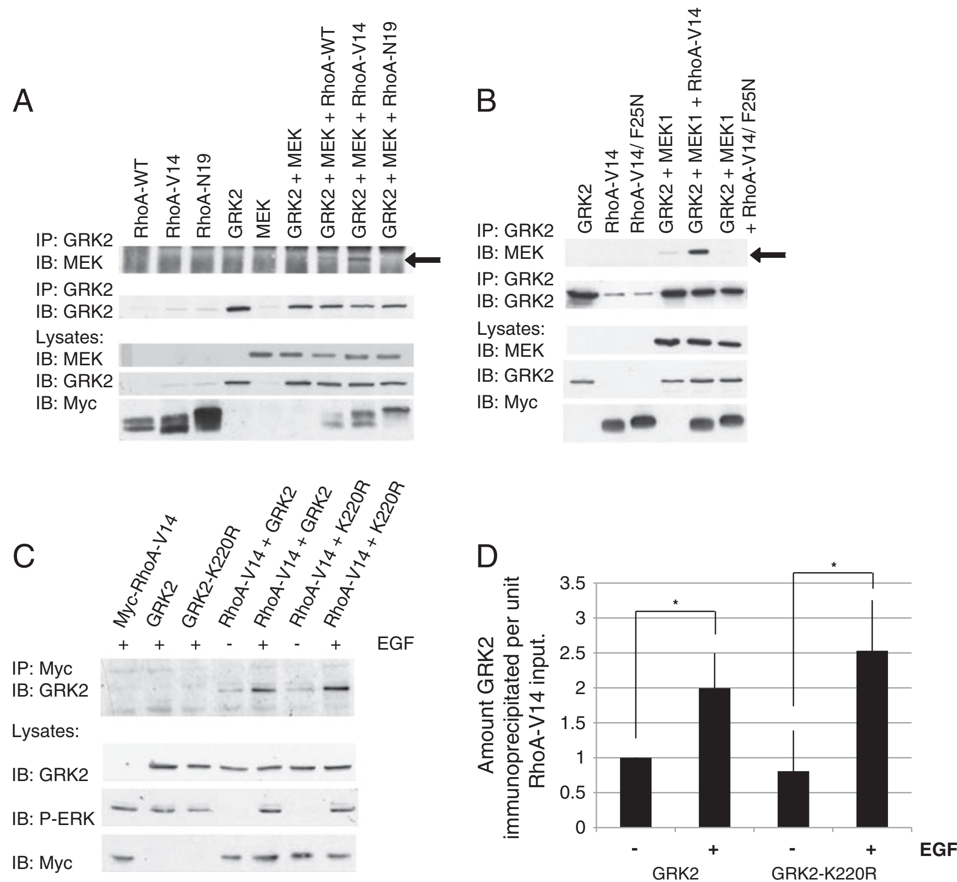


Fig. 4. EGF promotes RhoA-V14 binding by GRK2 and GRK2-K220R. COS cells were transfected as indicated. (A–B) Expression of RhoA mutants was confirmed by blotting with an anti-Myc antibody. GRK2 was immunoprecipitated (IP) and immunoprecipitates were probed (IB) with an anti-MEK antibody. (C) Cells were serum starved for 24 h and treated or not with EGF (200 ng/ml) for 5 min. Protein expression was confirmed by Western blotting (lysates) and Myc-tagged RhoA-V14 was immunoprecipitated (IP). Immunoprecipitates were probed (IB) with an anti-GRK2 antibody. The Western blots shown are representatives from 3 separate experiments. (D) Quantification of (C), amount of GRK2 co-immunoprecipitated with Myc-RhoA-V14 per unit Myc-RhoA-V14 expressed in lysates is plotted relative to amount of co-immunoprecipitation seen for wildtype GRK2, error bars represent standard deviation from the mean from 3 separate experiments, statistical significance was assessed by comparing samples treated with EGF with the relevant untreated controls, using the two-sample Student's *t*-test, *P < 0.05.

hypothesised that the Rho-mediated ERK scaffolding role of GRK2 that we have demonstrated in HEK-293 cells might function downstream of the EGFR in VSMCs.

We cultured VSMCs from rat aortas and performed thymidine incorporation experiments to measure EGF-induced VSMC proliferation. Treatment with EGF or serum for 24 h results in, respectively, a ~2 and ~3-fold increase in thymidine incorporation relative to untreated cells (Fig. 6A). Pre-treatment of VSMCs with the RhoABC inhibitor C3 or with the MEK1 inhibitor PD184352 resulted in 49 ± 13% and 62 ± 6.2% respective reductions in thymidine incorporation, indicating that VSMC proliferation is, at least in part, Rho and ERK dependent (Fig. 6B). Nucleofection of siRNA to GRK2 typically resulted in a ~70% reduction in GRK2 levels (Fig. 6C inset). EGF-induced thymidine incorporation in VSMCs depleted of GRK2 was reduced by 59 ± 12% relative to cells nucleofected with a scrambled control (Fig. 6C), indicating that GRK2 is also required for EGF-induced proliferation of VSMCs. We cannot detect GRK3 in these cells by Western blot (data not shown). This together with the observation that the MEK inhibitor and specific depletion of GRK2 produce a similar percentage inhibition of EGF-dependent thymidine incorporation suggests a specific role for GRK2, and not other GRKs, in EGFR and ERK-dependent thymidine incorporation in this model system. Taken together, this data demonstrates that GRK2, Rho and ERK are all required for VSMC proliferation in response to EGF, consistent with a role for Rho-activated ERK scaffolding by GRK2 downstream of the EGFR in VSMCs.

4. Discussion

We have identified a novel function for GRK2 as a Rho effector. Activated RhoA, which binds directly to the catalytic domain of GRK2, promotes the interaction of GRK2 with Raf1, MEK1 and ERK2, suggesting that GRK2 acts as a RhoA-activated ERK MAPK scaffold. Consistent with this hypothesis and in agreement with previously published data [10], we find that overexpression of GRK2 results in increased EGF-induced ERK activation. We show that this effect of GRK2 is Rho-dependent and that EGF treatment promotes the interaction between GRK2 and Rho_{GTP} and between GRK2 and P-ERK, in a kinase-independent fashion. Previously, EGF-mediated ERK activation was shown to be potentiated in HEK-293 cells by over-expression of phosphodiesterase-γ (PDEγ), a GRK2 substrate [36]. GRK2-mediated PDEγ phosphorylation in response to EGF can increase ERK activation by promoting the formation of a signalling complex containing GRK2 and Src [11]. In light of the kinase-independent ERK scaffolding function for GRK2 reported herein, we can conclude that GRK2 is able to potentiate EGF-mediated ERK activation by both kinase-dependent and independent mechanisms.

The Raf kinase inhibitor protein (RKIP) can inhibit the kinase activity of either Raf1 or GRK2 depending on its phosphorylation and oligomerisation state [37]. PKC phosphorylation of RKIP, and consequent dimerisation, dissociates RKIP from Raf1 and promotes binding to GRK2. This increases receptor-stimulated ERK activity due to disinhibition of

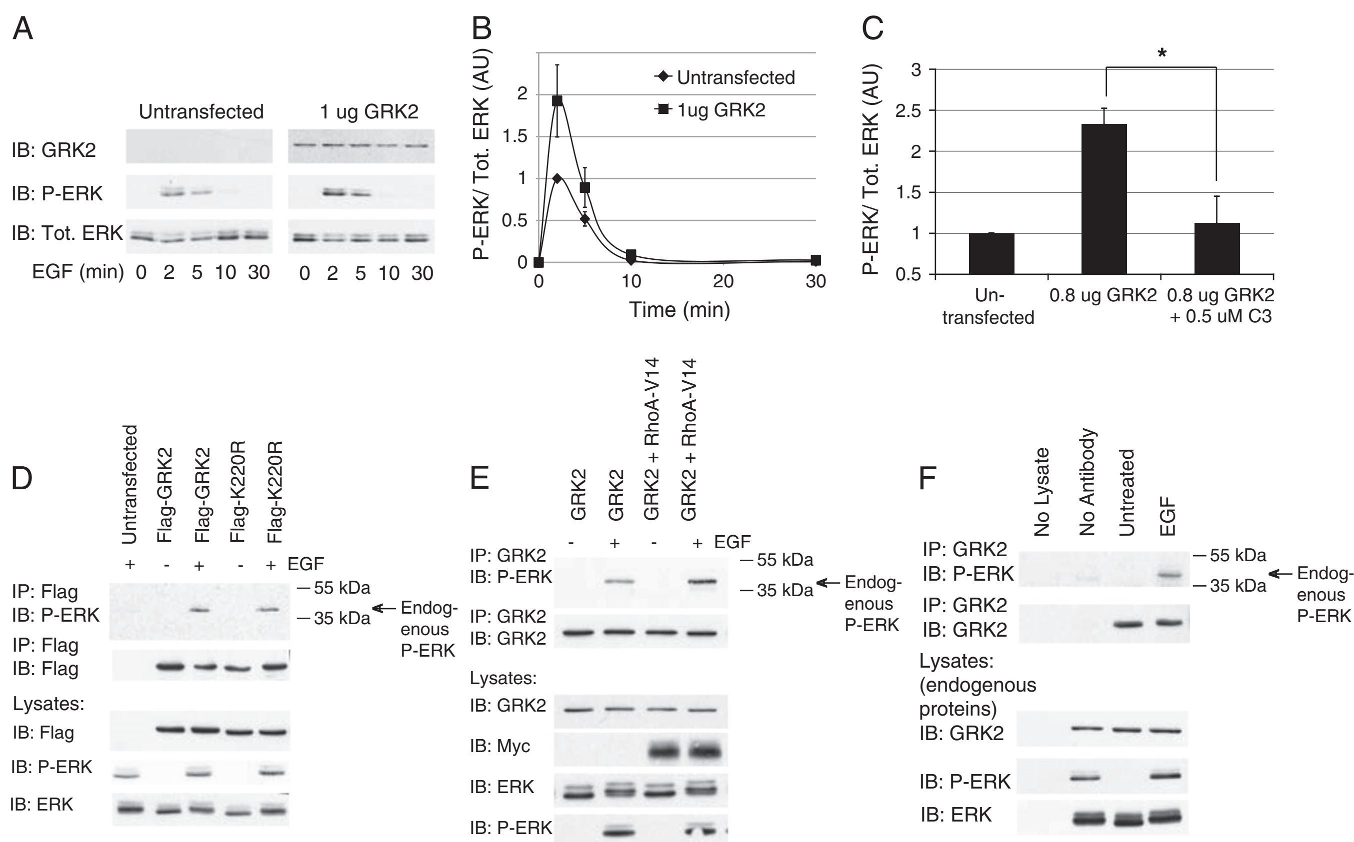


Fig. 5. GRK2 potentiates EGF-induced ERK activation in HEK-293 cells by acting as a Rho-dependent scaffold protein. (A–C) HEK-293 cells in 9 cm plates were transfected or not with the indicated amounts of GRK2. 24 h later, the cells were split onto 12-well plates and then serum starved overnight in the presence or absence of 0.5 μM C3 before treatment with EGF (200 ng/ml) for the times indicated (samples treated for 5 min in (C)). Lysates were blotted for levels of phospho- and total ERK and ERK activation was calculated by quantifying bands using Quantity One and dividing values for phospho-ERK by values for total ERK in each sample. (A) is a representative of 3 separate experiments, panels shown in (A) are from the same piece of nitrocellulose membrane to ensure equivalent transfer and exposure across different conditions. In (B) and (C), the fold increase in EGF-induced ERK activation in response to GRK2 transfection is plotted relative to untransfected cells. Error bars represent standard deviation from mean of 3 separate experiments, statistical significance was assessed using the two-sample Student's *t*-test, $^{*}P < 0.05$. (D) HEK-293 cells were transfected as indicated, serum starved overnight and treated or not with EGF (200 ng/ml) for 5 min. Protein expression was confirmed by Western blotting (lysates) with Flag-GRK2 constructs detected using an anti-Flag antibody. GRK2 was immunoprecipitated (IP) with an anti-Flag antibody and immunoprecipitates were probed (IB) for P-ERK. (E) HEK-293 cells were transfected as indicated, serum starved overnight and treated or not with EGF (200 ng/ml) for 5 min. Protein expression was confirmed by Western blotting (lysates) with Myc-RhoA-V14 detected using an anti-Myc antibody. GRK2 was immunoprecipitated (IP) and immunoprecipitates were probed (IB) for P-ERK. (F) HEK-293 cells were serum starved overnight and treated or not with EGF (200 ng/ml). Lysates were blotted for endogenous GRK2, ERK and P-ERK and GRK2 was immunoprecipitated (IP). Immunoprecipitates were probed (IB) with an anti-P-ERK antibody or an anti-GRK2-3 antibody to check IP efficiency. Western blots are representative of 3 separate experiments.

Raf1 as well as inhibition of GRK2-mediated receptor desensitisation [37,38]. It remains to be determined whether the kinase-independent ERK scaffolding function of GRK2 that we have described operates following RKIP binding and whether this contributes to the enhanced ERK activity observed following RKIP-mediated inhibition of GRK2 kinase activity.

β-arrestins, which function in concert with GRKs to desensitise GPCRs, are well-characterised MAPK scaffold proteins. GRK2-mediated and β-arrestin2-mediated scaffolding of the ERK MAPK cascade differs in several aspects. Firstly, the ability of GRK2, but not β-arrestin2, to bind components of the ERK cascade is stimulus ($RhoA_{GTP}$)-dependent. Secondly, GRK2-dependent ERK activation is rapid and transient (Fig. 5A and 5B), [10] whilst β-arrestin tends to promote sustained ERK activation [39]. Finally, β-arrestin scaffolding biases ERK towards its cytosolic substrates [40]. In VSMCs, GRK2 is required for EGF-induced thymidine incorporation, suggesting that ERK scaffolding by GRK2 may be important for nuclear rather than cytosolic functions of ERK. We are currently investigating the precise subcellular localisation of ERK activated downstream of GRK2.

Like GRK2, connector enhancer of KSR-1 (CNK1) also appears to function in a Rho-dependent manner. CNK1 is a scaffold protein that can regulate a number of different signalling pathways including PI3K/Akt activation, Src-mediated Raf1 activation and JNK MAPK signalling [41]. It has been identified as a target of Rho and it seems that binding of active Rho by CNK1 specifically mediates its scaffolding of the JNK MAPK cascade [42]. Thus it appears that CNK1 and GRK2 both use binding to active Rho to selectively regulate MAPK activation *versus* their other signalling functions.

GRK2 can in fact regulate ERK signalling in a number of different ways. In keeping with its classical role in GPCR desensitisation it negatively regulates ANG_{II}-induced ERK activation by phosphorylating and promoting internalisation of the receptor in HEK-293 cells [43] and *in vivo* [33]. It also co-immunoprecipitates with MEK [30] and various groups have suggested that sequestration of MEK by GRK2 is responsible for negative regulation of chemokine-mediated ERK activation [44,45]. Similarly, by binding and sequestering NFκB1p105, GRK2 can negatively regulate IKKβ- and TPL2-mediated ERK activation in response to lipopolysaccharide in mouse peritoneal macrophages [46]. Conversely, as well as downstream of EGF, GRK2 has also been shown to promote ERK activation downstream of sphingosine 1-phosphate in migrating epithelial cells by recruiting GIT, which in turn is proposed to activate MEK by scaffolding Rac/PAK/MEK [47].

Clearly GRK2 has a highly complex role in regulating MAPK signalling, as well as other signalling pathways, in both positive and negative ways. How such a multifunctional protein can achieve signalling

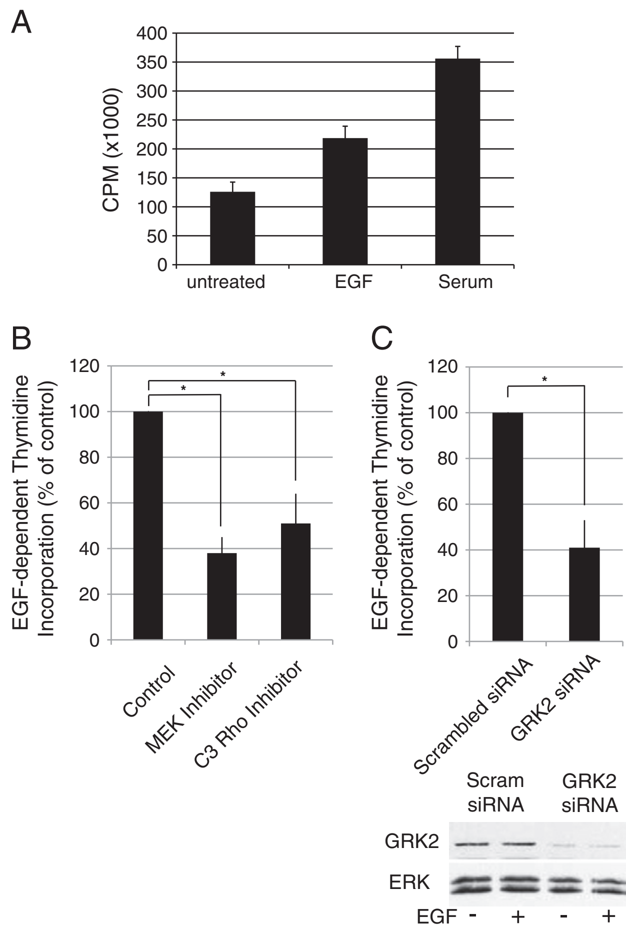


Fig. 6. GRK2 is required for EGF-induced proliferation of VSMCs. (A) VSMCs were serum starved for 24 h and treated with EGF (10 ng/ml) or serum (10%) for 24 h in the presence of [^{3}H]-thymidine (3 uCi/ml). (B) After 24 h serum starving, cells were treated with EGF (10 ng/ml) for a further 24 h in the presence of [^{3}H]-thymidine (3 uCi/ml) with or without pre-treatment with the MEK inhibitor PD184352 (1 uM) or the Rho inhibitor C3 (0.5 uM). (C) VSMCs were nucleofected or not with GRK2 siRNA or scrambled control (100 nM). After 24 h the cells were serum starved for 24 h and then treated with EGF (10 ng/ml) for a further 24 h in the presence of [^{3}H]-thymidine (3 uCi/ml) After 24 h EGF treatment, cells were harvested and counted for incorporation of [^{3}H]-thymidine. Fold increase in [^{3}H]-thymidine incorporation is plotted as a percentage of scrambled control in (B) or untreated control in (C). Error bars represent standard deviation from the mean of 3 separate experiments, statistical significance was assessed using the two-sample Student's *t*-test, *P < 0.01.

specificity downstream of different agonists or in different cell types is an interesting and poorly understood issue. Here we find that, like CNK1, GRK2 achieves specificity for ERK scaffolding by binding to active Rho, suggesting that ERK scaffolding by GRK2 may occur specifically downstream of receptors that activate Rho. In reality, however, the mechanisms of signalling specificity downstream of GRK2 are likely more complex. For example, while over-expression of GRK2 in HEK-293 cells results in increased EGF-induced ERK activation, as previously described, ERK activation in response to ANG_{II}, which also activates Rho, is inhibited by GRK2 over-expression in these cells [43]. Given that RhoA-V14 interacts with the catalytic domain of GRK2 three times more strongly than with full length GRK2, it is likely that a conformational change is required to expose the catalytic domain of GRK2 for full Rho_{GTP} binding *in vivo*. Therefore ERK scaffolding by GRK2 may occur downstream of receptors that both activate Rho and somehow modify GRK2 in order to expose its catalytic domain. GRK2 is a known EGFR substrate [48] suggesting one potential mechanism whereby the specificity of the Rho-dependent ERK scaffolding function of GRK2 may be regulated.

5. Conclusions

GRK2 is a novel Rho effector that interacts specifically and directly with active RhoA *via* its catalytic domain. In response to $RhoA_{GTP}$ binding, GRK2 binds to Raf1, MEK1 and ERK2. GRK2 promotes ERK activation in a Rho-dependent manner downstream of the EGFR and binding of both RhoA and phospho-ERK by GRK2 is increased in response to EGF treatment. Thus, RhoA-activated ERK scaffolding by GRK2 functions to promote ERK activation downstream of the EGFR. GRK2, Rho and ERK are all required for EGF-induced proliferation of VSMCs, consistent with a role for this novel scaffolding function of GRK2 in the vasculature.

Acknowledgements

This work was funded by grants from the Wellcome Trust (to JAP) and Medical Research Council (to JDR).

References

[1] J.A. Pitcher, N.J. Freedman, R.J. Lefkowitz, Annu. Rev. Biochem. 67 (1998) 653–692.
[2] C. Ribas, P. Penela, C. Murga, A. Salcedo, C. Garcia-Hoz, M. Jurado-Pueyo, I. Aymerich, F. Mayor Jr., Biochim. Biophys. Acta 1768 (2007) 913–922.
[3] R.T. Premont, R.R. Gainetdinov, Annu. Rev. Physiol. 69 (2007) 511–534.
[4] C.J. Hupfeld, J.M. Olefsky, Annu. Rev. Physiol. 69 (2007) 561–577.
[5] C. Waters, S. Pyne, N.J. Pyne, Semin. Cell Dev. Biol. 15 (2004) 309–323.
[6] N.J. Freedman, L.K. Kim, J.P. Murray, S.T. Exum, L. Brian, J.H. Wu, K. Peppel, J. Biol. Chem. 277 (2002) 48261–48269.
[7] J.H. Wu, R. Goswami, L.K. Kim, W.E. Miller, K. Peppel, N.J. Freedman, J. Biol. Chem. 280 (2005) 31027–31035.
[8] J. Gao, J. Li, Y. Chen, L. Ma, FEBS Lett. 579 (2005) 122–126.
[9] K.L. Hildreth, J.H. Wu, L.S. Barak, S.T. Exum, L.K. Kim, K. Peppel, N.J. Freedman, J. Biol. Chem. 279 (2004) 41775–41782.
[10] J. Gao, J. Li, L. Ma, Acta Biochim. Biophys. Sin. 37 (2005) 525–531.
[11] K.F. Wan, B.S. Sambi, R. Tate, C. Waters, N.J. Pyne, J. Biol. Chem. 278 (2003) 18658–18663.
[12] P. Penela, C. Murga, C. Ribas, V. Lafarga, F. Mayor Jr., Br. J. Pharmacol. 160 (2010) 821–832.
[13] S. Peregrin, M. Jurado-Pueyo, P.M. Campos, V. Sanz-Moreno, A. Ruiz-Gomez, P. Crespo, F. Mayor Jr., C. Murga, Curr. Biol. 16 (2006) 2042–2047.
[14] M. Ciccarelli, J.K. Chuprun, G. Rengo, E. Gao, Z. Wei, R.J. Peroutka, J.I. Gold, A. Gumpert, M. Chen, N.J. Otis, G.W. Dorn II, B. Trimarco, G. Iaccarino, W.J. Koch, Circulation 123 (2011) 1953–1962.
[15] J. Ho, E. Cocolakis, V.M. Dumas, B.I. Posner, S.A. Laporte, J.J. Lebrun, EMBO J. 24 (2005) 3247–3258.
[16] S.H. Cant, J.A. Pitcher, Mol. Biol. Cell 16 (2005) 3088–3099.
[17] S. Liu, R.T. Premont, C.D. Kontos, S. Zhu, D.C. Rockey, Nat. Med. 11 (2005) 952–958.
[18] D.M. Harris, H.I. Cohn, Clin. Sci. (Lond.) 115 (2008) 79–89.
[19] G.W. Dorn II, J. Mol. Med. (Berl.) 87 (2009) 455–463.
[20] L.R. Johnson, M.G. Scott, J.A. Pitcher, Mol. Cell. Biol. 24 (2004) 10169–10179.
[21] X. Jiang, P. Yang, L. Ma, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 10183–10188.
[22] R. McMullan, E. Hiley, P. Morrison, S.J. Nurrish, Genes Dev. 20 (2006) 65–76.
[23] A.J. Ridley, P.M. Comoglio, A. Hall, Mol. Cell. Biol. 15 (1995) 1110–1122.
[24] C.D. Nobes, A. Hall, Cell 81 (1995) 53–62.
[25] E. Sahai, M.F. Olson, Methods Enzymol. 406 (2006) 128–140.
[26] P. Rodriguez-Viciana, J. Oses-Prieto, A. Burlingame, M. Fried, F. McCormick, Mol. Cell 22 (2006) 217–230.
[27] S. Govindan, E.J. Taylor, C.W. Taylor, Br. J. Pharmacol. 160 1953–1962.
[28] A.P. Wheeler, A.J. Ridley, Exp. Cell Res. 301 (2004) 43–49.
[29] R.T. Premont, A. Claing, N. Vitale, J.L. Freeman, J.A. Pitcher, W.A. Patton, J. Moss, M. Vaughan, R.J. Lefkowitz, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 14082–14087.
[30] M.C. Jimenez-Sainz, C. Murga, A. Kavelaars, M. Jurado-Pueyo, B.F. Krakstad, C.J. Heijnen, F. Mayor Jr., A.M. Aragay, Mol. Biol. Cell 17 (2006) 25–31.
[31] A. Elorza, S. Sarnago, F. Mayor Jr., Mol. Pharmacol. 57 (2000) 778–783.
[32] M.R. Schiller, Cell. Signal. 18 (2006) 1834–1843.
[33] A.D. Eckhart, T. Ozaki, H. Tevaearai, H.A. Rockman, W.J. Koch, Mol. Pharmacol. 61 (2002) 749–758.
[34] A. Yogi, S.E. O'Connor, G.E. Callera, R.C. Tostes, R.M. Touyz, Curr. Opin. Nephrol. Hypertens. 19 (2010) 169–176.
[35] G. Loirand, P. Pacaud, Nat. Rev. Cardiol. 7 (2010) 637–647.
[36] K.F. Wan, B.S. Sambi, M. Frame, R. Tate, N.J. Pyne, J. Biol. Chem. 276 (2001) 37802–37808.
[37] K. Deiss, C. Kisker, M.J. Lohse, K. Lorenz, J. Biol. Chem. 287 (2012) 23407–23417.
[38] K. Lorenz, M.J. Lohse, U. Quitterer, Nature 426 (2003) 574–579.
[39] S. Ahn, S.K. Shenoy, H. Wei, R.J. Lefkowitz, J. Biol. Chem. 279 (2004) 35518–35525.
[40] S.M. DeWire, S. Ahn, R.J. Lefkowitz, S.K. Shenoy, Annu. Rev. Physiol. 69 (2007) 483–510.
[41] A. Ziogas, K. Moelling, G. Radziwill, J. Biol. Chem. 280 (2005) 24205–24211.
[42] A.B. Jaffe, A. Hall, A. Schmidt, Curr. Biol. 15 (2005) 405–412.

[43] J. Kim, S. Ahn, X.R. Ren, E.J. Whalen, E. Reiter, H. Wei, R.J. Lefkowitz, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 1442–1447.
[44] W. Kleibeuker, M. Jurado-Pueyo, C. Murga, N. Eijkelkamp, F. Mayor Jr., C.J. Heijnen, A. Kavelaars, J. Neurochem. 104 (2008) 979–992.
[45] M. Jurado-Pueyo, P.M. Campos, F. Mayor, C. Murga, J. Recept. Signal Transduct. Res. 28 (2008) 59–70.
[46] S. Patial, Y. Saini, S. Parvataneni, D.M. Appledorn, G.W. Dorn 2nd, J.J. Lapres, A. Amalfitano, P. Senagore, N. Parameswaran, J. Cell. Physiol. 226 (2011) 627–637.
[47] P. Penela, C. Ribas, I. Aymerich, N. Eijkelkamp, O. Barreiro, C.J. Heijnen, A. Kavelaars, F. Sanchez-Madrid, F. Mayor Jr., EMBO J. 27 (2008) 1206–1218.
[48] Y. Chen, H. Long, Z. Wu, X. Jiang, L. Ma, Mol. Biol. Cell 19 (2008) 2973–2983.