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The mechanism and function of mitogen-activated protein kinase activation by ARF1

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Abstract

Mitogen-activated protein kinases (MAPK) can be activated by a number of biochemical pathways through distinct signaling molecules. We have recently revealed a novel function for the Ras-like small GTPase ADP-ribosylation factor 1 (ARF1) in mediating the activation of Raf1-MEK-ERK1/2 pathway by G protein-coupled receptors. Here, we have further defined the underlying mechanism and the possible function of ARF1-mediated MAPK pathway. We demonstrated that the blockage of ARF1 activation and the disruption of ARF1 localization to the Golgi by mutating Thr48, a highly conserved residue involved in the exchange of GDP for GTP, and the myristoylation site Gly2 abolished ARF1's ability to activate ERK1/2. In addition, treatment with Golgi structure disrupting agents markedly attenuated ARF1-mediated ERK1/2 activation. Furthermore, ARF1 significantly promoted cell proliferation. More interestingly, ARF1 activated 90kDa ribosomal S6 kinase 1 (RSK1) without influencing Elk-1 activation and ERK2 translocation to the nuclei. These data demonstrate that, once activated, ARF1 activates the MAPK pathway likely using the Golgi as a main platform, which in turn activates the cytoplasmic RSK1, leading to cell proliferation.

1. Introduction

Mitogen-activated protein kinases (MAPKs) regulate a variety of cell functions and can be activated by a diverse array of extracellular stimuli and through distinct biochemical pathways. The best characterized MAPK signaling cascade involves the activation of the cell surface growth factor receptors, activation of cytoplasmic signaling proteins and phosphorylation/activation of three protein kinases, Raf1, MEK and ERK1/2 [1, 2]. Over the past decades, a number of signaling molecules, including protein kinases, heterotrimeric G

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proteins, small GTPases, and β-arrestins, have been identified to be involved in the activation of the Raf1-MEK-ERK1/2 pathway by G protein-coupled receptors (GPCRs) and predominant mechanism used depends upon receptor and cell types [3-10].

ADP-ribosylation factors (ARFs) are Ras-related small GTPases and have been well characterized to regulate vesicular trafficking. Specifically, ARF1 plays a crucial role in the formation of distinct transport vesicles in both anterograde and retrograde transport pathways [11-18]. Like other GTPases, the function of ARF1 is highly regulated by its recycling between active GTP-bound and inactive GDP-bound conformation. The GDP-bound ARF1 may be recruited from cytosol onto the membrane by interacting with receptor proteins and its association with the membrane is mediated through the N-terminal myristoylated amphipathic helix domain, a process important for the exchange of GDP for GTP by guanine nucleotide exchange factors. Active GTP-bound ARF1 subsequently interacts with downstream effectors to recruit distinct protein complexes onto different intracellular compartments, resulting in the formation of different transport vesicles. In addition, ARF1 has been shown to activate signaling molecules, such as phospholipase D, PI3K and type I phosphatidylinositol 4-phosphate 5-kinase [19-26]. These data suggest that, in addition to its well-established trafficking function, ARF1 GTPase also functions as a signal transducer

During the studies of regulation of GPCR trafficking and signaling by ARFs [27, 28], we found that the activation the MAPK Raf1-MEKERK1/2 pathway by α_{2B}-adrenergic receptor (α_{2B}-AR), a typical GPCR, may be partially mediated through ARF1 [29]. In the current study, we have further defined the underlying molecular mechanism and the possible function of this ARF1-mediated MAPK signaling pathway.

2. Experimental procedures

2.1. Materials

The glutathione S-transferase (GST) fusion protein constructs coding the VHS and VHS-GAT domains of Golgi-localized γ-ear-containing ARF1-binding protein 3 (GGA3) and the GFP-ERK2 construct were provided by Dr. Juan S. Bonifacino and Dr. Philip J.S. Stork, respectively. RSK1 dominant-negative mutant (pKH3-avian RSK1K112/464R) was obtained from Addgene (Cambridge, MA). ARF1 antibodies were purchased from Stressgen (Ann Arbor, MI). Antibodies against green fluorescent protein (GFP) and phospho-ERK1/2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against ERK1/2 and cyclin D1 were from Cell Signaling Technology, Inc. (Beverly, MA). Giantin antibodies were from Abcam Inc. (Cambridge, MA); GW5074, wortmannin and morin were from Sigma (St Louis, MO). U0126, PD98059, PP2, KT5720, KT5823 and LY294002 were purchased from Calbiochem (San Diego, CA). Nacodazole, brefeldin A (BFA) and ilimaquinone were from Sigma (ST Louis, MO). [^{3}H]-RX821002 (specific activity = 50 Ci/mmol) was from Perkin Elmer Life Sciences. All other materials were obtained as described elsewhere [29-31].

2.2. Plasmid constructions

ARF1 and its mutants conjugated with GFP at their C-termini were generated by using the BamHI and EcoRI restriction sites of the pEGFP-N1 vector. The mutations were carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequence of each construct used in this study was verified by restriction mapping and nucleotide sequence analysis.

2.3. Cell culture and transient transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transient transfection of the HEK293 cells was carried out using Lipofectamine 2000 reagent (Invitrogen) as previously described [33]. The transfection efficiency was estimated to be greater than 70% based on the GFP fluorescence.

2.4. Measurement of ARF1 activation

The GST pull down assay was used to measure ARF1 activation by using GST-VHS- GAT- fusion protein in which the GAT domain specifically interacts with the activated form of ARF1 as described [32]. GST fusion proteins were expressed in bacteria and purified using a glutathione affinity matrix as described previously [33]. GST fusion proteins immobilized on the glutathione resin were incubated with total lysate prepared from cells transfected with ARF1 or its mutants in 500 μl of binding buffer (20 mM Tris-HCl, pH 7.5, 2% NP-40, 70 mM NaCl) at 4 °C overnight. The resin was washed four times with 1 ml of binding buffer, and the retained proteins were solubilized in 1 × SDS gel loading buffer and separated by SDS-PAGE. Bound proteins were detected by immunoblotting.

2.5. Measurement of ERK1/2 and RSK1 activation

HEK293 cells cultured on 6-well dishes were transfected with 1.0 μg of ARF1 for 8 h. The cells were then split at 1:2 ratio and cultured for additional 18-24 h. For drug treatment, HEK293 cells were transfected with ARF1Q71L and then treated with individual drug overnight. The cells were solubilized by addition of 300 μl SDS gel loading buffer and 20 μl of total cell lysate was separated by SDS-PAGE. Activation of ERK1/2 and RSK1 was determined by immunoblotting measuring their phosphorylation with phosphor-specific antibodies as described previously [29, 30].

2.6. Measurement of Elk-1 activation

Elk-1 activation was measured by using the Elk-1-driven luciferase reporter system (Stratagene, La Jolla, CA) as described [34]. HEK293 cells cultured on 6-well dishes were transfected with 0.5 μg ARF1Q71L together with 25 ng GAL4-Elk-1, 0.5 μg pFR-luc. After 6 h, the cells were split onto 12-well dishes. Twenty-four h after transfection, the cells were starved for 6 h and lysed with 100 μl of luciferase lysis buffer (Promega). Five μl of total cell lysates was used to measure luciferase activities using a luminometer (Model 2010, MGM Instrument).

2.7. Confocal Fluorescence Microscopy

HEK293 cells grown on coverslips pre-coated with poly-L-lysine in 6-well plates were transfected with ARF1 for 36 h. The cells were then fixed with 4% paraformaldehyde-4% sucrose mixture in PBS for 15 min, permeabilized with PBS containing 0.2% Triton X-100 for 5 min, and blocked with 5% FBS for 1 h. The cells were then incubated with antibodies against giantin, a Golgi marker, at a dilution of 1:500 for 1 h. After washing with PBS (3 × 5 min), the cells were incubated with Alexa Fluor 594-labeled secondary antibody (1:1000 dilution) for 1 h at room temperature. To visualize the subcellular localization of ERK2, ERK2 was tagged with GFP and co-transfected with ARF1Q71L in HEK293 cells. The coverslips were mounted with prolong antifade reagent (Invitrogen) and images were captured using a LSM510 Zessis confocal microscope equipped with a 63× objective as described previously [35]

2.8. Intact cell radioligand binding

The effect of ARF1 on the cell surface expression of α_{2B}-AR was measured by ligand binding of intact live cells using HEK293 cells stably expressing HA-α_{2B}-AR and the membrane impermeable ligand [^{3}H]-RX821002 as described previously [35, 36]. Briefly, HEK293 cells cultured on 6-well dishes were transfected with ARF1 and then split into 12-well dishes. The cells were incubated with 300 μl of DMEM plus [^{3}H]-RX821002 at a concentration of 20 nM for 90 min at room temperature. The cells were washed twice with 1 ml of ice-cold PBS and the cell surface-bound ligands extracted by 1 M NaOH treatment for 2 h. The non-specific binding was determined in the presence of rauwolscine (10 μM). The radioactivity was counted by liquid scintillation spectrometry in 3.5 ml of Ecoscint A scintillation solution.

2.9. Measurement of cell proliferation

Cell proliferation was measured by carboxyfluorescein diacetate duccinimidyl ester (CFSE) staining as described [37]. In this method, the reduction in the intensity of CFSE staining would reflect the enhancement of cell division. HEK293 cells were transfected with ARF1Q71L with or without cotransfection of the dominant negative mutant RSK1K112/464R for 18 h. The cells were washed with HBSS and stained with 5 μM CFSE in 1 ml HBSS at 37°C for 5 min. After replating the cells on 6-well dishes at a density of 75 $\times 10^4$ cells/well and culture for 4 days, the cells were then harvested and analyzed by flow cytometry.

2.10. Immunoblotting

The total cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The signal was detected using ECL Plus (PerkinElmer Life Sciences) and a Fuji Film luminescent image analyzer (LAS-1000 Plus) and quantitated using the Image Gauge program (Version 3.4).

2.11. Statistical analysis

Differences were evaluated using Student's *t* test, and $p < 0.05$ was considered as statistically significant. Data are expressed as the mean ± S.E.

3. Results

3.1. Effects of various protein kinase inhibitors on ARF1-mediated ERK1/2 activation

In additional to Raf1 and MEK, several other protein kinases, including Src, protein kinase A (PKA), protein kinases C (PKC), phosphoinositide 3-kinases (PI3K) and phosphatidylinositol 4-phosphate kinases (PIP4K) have been shown to participate in the activation of ERK1/2 by different stimuli [38]. To determine if these protein kinases are involved in ERK1/2 activation by ARF1, HEK293 cells were transiently transfected with ARF1Q71L, a constitutively active GTP-bound ARF1 mutant, and then treated with various kinase inhibitors. Consistent with our previous data [29], transient expression of ARF1Q71L markedly enhanced the activation of ERK1/2 pathway in the absence of extracellular stimuli. Treatments with the Raf1 inhibitor GW5074 and the MEK inhibitors U0126 and PD98059 profoundly blocked the ERK1/2 activation by ARF1Q71L (Fig. 1A and 1B). In contrast, treatments with PP2 (a Src inhibitor), KT5720 (a PKA inhibitor), KT5823 (a PKC inhibitor), LY294002, wortmannin (PI3K inhibitors), and morin (a PIP4K inhibitor) at concentrations which have been previously shown to inhibit ERK1/2 activation did not significantly alter ERK1/2 activation by ARF1Q71L (Fig. 1A and 1B). These data suggest that the protein kinases Src, PKC, PKA, PI3K and PIP4K are unlikely involved in ARF1-mediated ERK1/2 activation.

3.2. Effect of mutating Thr48 on ARF1 activation and ARF1-mediated activation of ERK1/2

Our previous studies via expressing through expressing the GTP-bound ARF1 mutant and ARF1 guanine nucleotide exchange factor ARNO [29] suggest that activated ARF1 is able to regulate the MAPK pathway. To further study if ARF1-mediated MAPK activation depended on ARF1 activation, we determined the effect of mutating Thr48, which is a highly conserved in switch I region of all small GTPases (Fig. 2A) and heterotrimeric G proteins and plays a crucial role in their activation [39-43]. The GTP-bound mutant ARF1Q71L was mainly studied in these experiments as it strongly activated the MAPK pathway. We first determined the effect of mutating Thr48 to Ser on ARF1Q71L activation by GST-VHS-GAT fusion protein pulldown assays (Fig. 2B) [32] and the subcellular localization of ARF1 by confocal microscopy. As expected, GTP-bound mutant ARF1Q71L strongly interacted with the VHS-GAT domain, but not the VHS domain and GST alone and the interaction of the GDP-bound mutant ARF1T31N with the VHS-GAT domain was much weaker than ARF1Q71L (Fig. 2C and 2D). Mutation of Thr48 dramatically attenuated ARF1Q71l interaction with the VHS-GAT domain (Fig. 2C and 2D). Consistent with other reports [17, 44], ARF1Q71L was extensively colocalized with the Golgi marker giantin (Fig. 2E), suggestive of predominant Golgi localization. Mutation of Thr48 clearly reduced ARF1Q71L localization to the Golgi and the double mutant ARF1Q71L/T48S was largely expressed in the cytoplasm (Fig. 2F).

To determine if mutation of Thr-48 could influence the trafficking function of ARF1, we measured its effect on the cell surface transport of α_{2B}-AR. Consistent with our previous report [27], transient expression of ARF1Q71l markedly inhibited the cell surface expression of α_{2B}-AR (Fig. 3A). Mutation of Thr48 dramatically reversed the ability of ARF1Q71L in blocking the cell surface transport of α_{2B}-AR (Fig. 3A).

We then determined the effect of mutating Thr48 on ARF1Q71L-mediated activation of ERK1/2. The activation of ERK1/2 was substantially lower in cells expressing ARF1Q71L/T48S than those in cells expressing ARF1Q71L (Fig. 3B and 3C). Consistent with our previous data, expression of ARF1 slightly enhanced the activation of ERK1/2 and the mutation of Thr48 to Ser reduced ARF1's ability to activate ERK1/2 (Fig. 3B and 3C). These data demonstrate that mutation of Thr48 to Ser in switch I region blocks ARF1 activation and reduces its abilities to activate ERK1/2.

3.3. Effect of mutating Gly2 on ARF1 activation and ARF1-mediated activation of ERK1/2

We next determined the effect of mutating Gly2 to Ala on the subcellular localization and activation of ARF1 and ARF1-mediated MAPK activation. It has been shown that ARF1 tethering to the membranes is mediated through myristoylation at Gly-2 and the myristoylation plays an important role in the activation of ARF1 [45-48]. The double mutant ARF1Q71L/G2A was completely unable to localize onto the Golgi and exclusively expressed in the cytoplasm, confirming the function of Gly-2 in ARF1 association with the Golgi (Fig. 4A). The mutation of Gly2 also dramatically inhibited the interaction of ARF1 with the VHS-GAT domain as measured in the GST fusion protein pulldown assay (Fig. 4B and 4C), indicating that the mutation prevents the activation of ARF1.

Similar to the effect of mutating Thr48, mutation of Gly2 substantially rescued the inhibitory effect of ARF1Q71L on the cell surface expression of α_{2B}-AR (Fig. 5A). We next determined the effect of mutation of Gly2 on ARF1-mediated activation of the MAPK pathway. Mutation of Gly2 abolished ARF1Q71L's ability to activate ERK1/2 (Fig. 5B and 5C). These data suggest that ARF1 activation as well as its localization to the Golgi are required for ARF1 to activate the MAPK pathway

3.4. Inhibition of ARF1-mediated activation of ERK1/2 by Golgi structure disruptors

Our preceding data demonstrated that GTP-bound ARF1Q71L strongly localized to the Golgi (Fig. 2E and 3A) and that mutation of Gly2 to Ala not only disrupted ARF1Q71L association with the Golgi but also abolished ARF1Q71L-mediated ERK1/2 activation. These data suggest that ARF1-mediated ERK1/2 may occur on the Golgi. To further test this possibility, we determined the effect of treatment with nacodazole, brefeldin A and ilimaquinone, three well-known Golgi structure disrupting agents, on the ERK1/2 activation by ARF1Q71L. The treatment with each of these Golgi disruptors significantly attenuated ERK1/2 activation in cells expressing ARF1Q71L (Fig. 6A and 6B). These data indicate that the intact Golgi structure may be required for ERK1/2 activation by ARF1Q71L and suggest that ARF1-mediated ERK1/2 activation likely occurs on the Golgi apparatus.

3.5. ARF1 enhances cell proliferation

We next sought to define the functional consequence of the MAPK activation by ARF1. As ARF1 is well-characterized trafficking protein, we first determined if the MAPK activation is essential for ARF1 function in regulating receptor transport. The treatment with the MAPK pathway inhibitors U0126 and PD98059 did not alter α_{2B}-AR transport to the plasma membrane in cells with or without transfection of ARF1Q71L (Fig. 7A). Similar results were obtained when the RasG12V mutant which activates the MAPK pathway was

tested (Fig. 7A). These data suggest that ARF1-mediated MAPK activation is unlikely involved in the regulation of ARF1 function as a transport modulator, at least in the cell surface export of α_{2B}-AR.

We then asked if ARF1-mediated activation of MAPK-RSK1 pathway can induce cell proliferation in HEK293 cells. Expression of cyclin D1, a positive regulator of the cell cycle, was increased by 2.5 folds by expression of ARF1Q71L (Fig. 7B and 7C). Cell proliferation was also significantly augmented in cells expressing ARF1Q71L as measured by CFSE staining (Fig. 7D). These data indicate that ARF1 is able to facilitate cell proliferation

3.6. Effect of ARF1 on the activation of Elk-1 and RSK1 and subcellular localization of ERK2

To dissecting the downstream effectors of ARF1-mediated ERK1/2 activation, we determined if the active mutant ARF1Q71 could activate Elk-1 and RSK1, two well-defined ERK1/2 substrates in the nucleus and cytoplasm, respectively [34, 49, 50]. Expression of the active mutant RasG12V, which was used as a positive control, markedly enhanced the activation of Elk-1, whereas expression of ARF1Q71L did not have obvious effect (Fig. 8A). Consistent with this, expression of ARF1Q71L did not induce the translocation of GFP-tagged ERK2 to the nucleus (Fig. 8B) and ERK2 was confined in the cytoplasm in cells expressing ARF1Q71L.

In marked contrast to its effect on the activation of Elk-1, expression of ARF1Q71L significantly increased the activation of RSK1, whereas expression of double mutants ARF1Q71L/T48S and ARF1Q71L/G2A which were inactive and unable to attach to the Golgi did not have clear effect (Fig. 7C and 7D). Furthermore, ARF1Q71L-mediated RSK1 activation was completely reversed by U0126 and PD98059 (Fig. 7E and 7F), suggesting that ARF1 activates RSK1 through the MAPK pathway.

To further determine if RSK1 activation is indeed responsible for enhanced cell proliferation by ARF1, we determined the effect of transient expression RSK1K112/464R, a dominant-negative RSK1 mutant [51], on cell proliferation by CFSE staining in cells transfected with ARF1Q71L. Cell proliferation was strongly increased in cell expressing ARF1Q71L, whereas expression of inactive mutants ARF1Q71L/T48S and ARF1Q71L/G2A had not clear effect on cell proliferation (Fig. 8G). ARF1Q71L-potentiated cell proliferation was largely inhibited by expressing RSK1K112/464R (Fig. 8G). These data suggest that the function of ARF1 in promoting cell proliferation is mediated through activating RSK1.

4. Discussion

A number of signaling molecules have been described to be involved in activation of the MAPK Raf-MEK-ERK1/2 pathway. Our recent studies have identified a novel function for the Ras-like small GTPase ARF1 in the activation of this pathway by the G protein-coupled α_{2B}-AR which is mediated through a specific interaction between the third intracellular loop of the receptors and ARF1 [29]. We have also shown that transient expression of the constitutively active, GTP-bound ARF1 mutant Q71L and expression of ARNO to facilitation of GDP exchange for GTP can potentiate the activation of the MAPK ERK1/2

pathway in the absence of any extracellular stimuli [29]. Here, we have utilized two different strategies to inactivate ARF1 to further explore the role of ARF1 activation in promoting the MAPK ERK1/2 pathway. The first strategy was to mutate Thr48, a highly conserved residue in both monomeric and heterotrimeric GTP-binding protein families and important for their activation [39-43]. Indeed, mutation of Thr48 markedly reduced the ability of ARF1Q71L to maintain its activation state as measured by interaction with the GAT domain of GGA. These data are consistent with previous studies showing an important role of Thr48 in the exchange of GDP for GTP of ARF1 [39-43]. Importantly, we have shown that mutating Thr48 abolished the ability of ARF1 to activate ERK1/2. The second strategy to block ARF1 activation was via mutating the myristoylation site Gly2. Similar to mutating Thr48, mutating Gly2 almost completely inhibited ARF1 activation and ARF1-mediated ERK1/2 activation. These results, together our previous data showing that the expression of ARF1 guanine nucleotide exchange factor ARNO promotes ERK1/2 activation [29], strongly demonstrate that the activation of the MAPK ERK1/2 pathway by ARF1 depends on the activation of ARF1.

Spatial and temporal regulation of the MAPK signaling pathway has provided fine-tuned mechanisms for a variety of cellular processes [6, 52]. For example, although Ras-mediated activation of the MAPK pathway has been considered to primarily occur on the plasma membrane, subcellular compartmentalization of the Ras signaling down to the MAPK pathway on the Golgi, the ER, endosomes and mitochondria has now been well documented [52, 53]. Our data presented in this manuscript suggest that the Golgi apparatus may spatially provide a main platform upon which ARF1 performs its function to activate the MAPK ERK1/2 pathway (Fig. 9). First, consistent with many other reports [17, 44], the GTP-bound mutant ARF1Q71L which strongly activates the MAPK pathway extensively localizes to the Golgi. Second, mutation of Thr48 to block ARF1 activation remarkably attenuate the Golgi localization of ARF1 and ERK1/2 activation by ARF1. Third, mutation of the myristoylation site Gly2 to destroy ARF1 association with the Golgi abolish ERK1/2 activation by ARF1. Fourth, the treatment with three well-known Golgi structure disruptors, which induce the Golgi disassembly through distinct mechanisms, strongly reduce the activation of ERK1/2 by ARF1.

ARF1 has been known as a key player involved in vesicle-mediated transport between intracellular organelles [11, 12, 54]. Indeed, we have previously demonstrated that expression of constitutively active and dominant negative mutants of ARF1 markedly inhibit the cell surface transport of several GPCRs, including α_{2B}-AR [27]. However, our current studies demonstrated that MAPK pathway inhibitor did not reverse the inhibitory effect of ARF1Q71L on α_{2B}-AR transport, suggesting that ARF1-mediated MAPK pathway unlikely plays a role in regulating its trafficking function, at least in the case of the cell surface transport of α_{2B}-AR.

Because the MAPK ERK1/2 pathway has been well described to regulate cell proliferation, to define the physiologic function of ARF1-mediated activation of the MAPK-RSK1 pathway, we determined the effect of ARF1Q71L on the expression of cyclin D1, a protein required for cell progression through the G1 phase of cell cycle, and CFSE staining of cells. We found that expression of ARF1Q71 significantly enhanced the expression of cyclin D1

and reduced CFSE staining, suggestive of augmentation of cell proliferation. Therefore, ARF1-mediated activation of Raf1-MEK-ERK1/2-RSK1 provides a novel signal transduction pathway to critically control cell proliferation.

Over the past decades, comprehensive studies on the molecular mechanisms underlying the activation of the Raf1-MEK-ERK1/2 pathway have revealed that Ras is a strong activator of the pathway [55, 56]. Although ARF1 and Ras, two closed-related small GTPases, are able to activate the MAPK pathway, the ultimate functional destination of the activated ERK1/2 by ARF1 is strikingly different from those by Ras as well as heterotrimeric G proteins. ERK1/2 activated by Ras and heterotrimeric G proteins translocate not only to the nucleus but also the cytosol to activate a number of transcriptional factors, such as Elk and RSK, and regulate diverse programs of gene transcription [49]. We demonstrated that expression of ARF1Q71L markedly activated RSK1, a well-characterized cytosolic subtract of the MAPK ERK1/2 pathway, but did not influence the activation of Elk, an ERK1/2 subtract in the nuclei. Consistently, expression of ARF1Q71L did not cause the translocation of ERK2 to the nuclei. Instead, ERK2 was confined in the cytoplasm in cells expressing ARF1Q71L. These data suggest that ARF1-activated ERK1/2 remain in the cytosol where they can activate the downstream effectors, such as RSK1 (Fig. 9).

The experiment results presented here, together with our previous data [29], have established a model in which, once activated, ARF1 leads to the activation of conventional Raf1-MEK-ERK1/2 pathway, which in turn activates the cytosolic RSK1 (which will presumably translocate to the nuclei), promoting cell proliferation (Fig. 9). However, it should be pointed out that there are many unanswered questions related to the activation of the MAPK pathway by activated ARF1, such as the followings: 1) How does ARF1 translocate to the Golgi upon activation by receptors located at the cell surface? 2) How does ARF1 activate the Raf1-MEK-ERK1/2 pathway? 3) What is the relationship between the Ras- and ARF1-mediated MAPK pathways? Nevertheless, we have identified an important signaling pathway that is mediated through the activation of the Ras-like small GTPase ARF1 to regulate cell proliferation, which is likely distinct from Ras-mediated activation of MAPK pathway. As ARF1 can be activated by many GPCRs and protein tyrosine kinase receptors [26, 29, 57-59], this pathway may be commonly used by these receptors to regulate cell growth under physiologic and/or pathological conditions (Fig. 9).

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The abbreviations used are

GPCR	G protein-coupled receptor
MAPK	mitogen-activated protein kinase
ERK1/2	extracellular signal-regulated kinase 1 and 2

ARF1	ADP-ribosylation factor 1
GGA	Golgi-localized γ-ear-containing ARF1-binding protein
GST	glutathione S-transferase
GFP	green fluorescent protein
DMEM	Dulbecco's modified Eagle's medium
PBS	phosphate-buffered saline

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Highlights

ARF1 activates the MAPK pathway in an activation-dependent manner.

ARF1-mediated activation of MAPK pathway likely uses the Golgi as a platform.

ARF1 activates the cytosolic RSK1.

ARF1-mediated activation MAPK-RSK1 pathway regulates cell proliferation.

A novel ARF1-mediated signaling pathway is proposed.

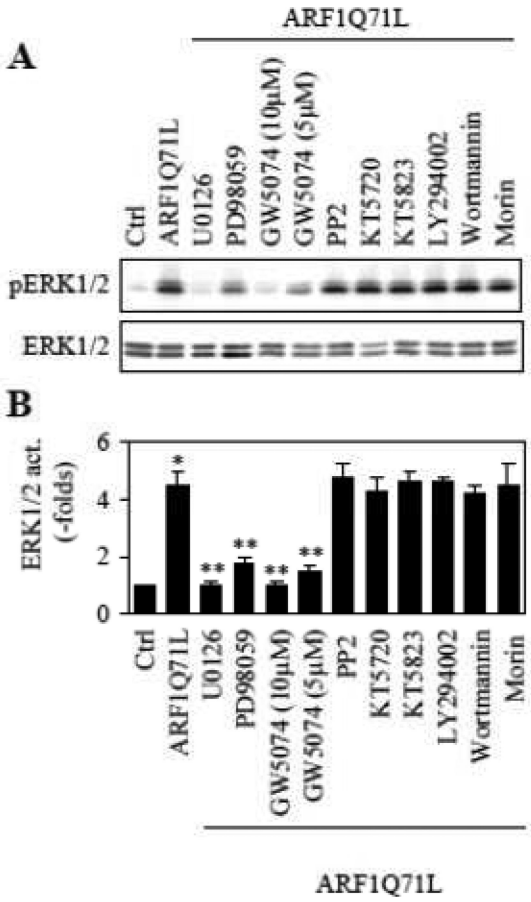

Fig. 1.

Effect of different protein kinase inhibitors on ARF1-mediated ERK1/2 activation. A, HEK293 cells were transiently transfected with the pcDNA3 vector (control) or the constitutively active GTP-bound mutant ARF1Q71L and then incubated overnight with U0126 (10 μM), PD98059 (50 μM), GW5274 (5 and 10 μM), PP2 (10 μM), KT5720 (1 μM), KT5823 (15 μM), LY294002 (50 μM), wortmannin (10 μM), and morin (50 μM). ERK1/2 activation was measured by Western blotting measuring their phosphorylation using phosphor-specific ERK1/2 antibodies as described under “Experimental Procedures”. Upper panel, a representative blot of ERK1/2 activation; lower panel, total ERK1/2 expression. B, Quantitative results of ERK1/2 activation. The data are expressed as –folds increase over the basal level and presented as mean ± S.E. of 3-6 experiments. *and **, $p < 0.05$ versus control (ctrl) and ARF1Q71L without drug treatment, respectively.

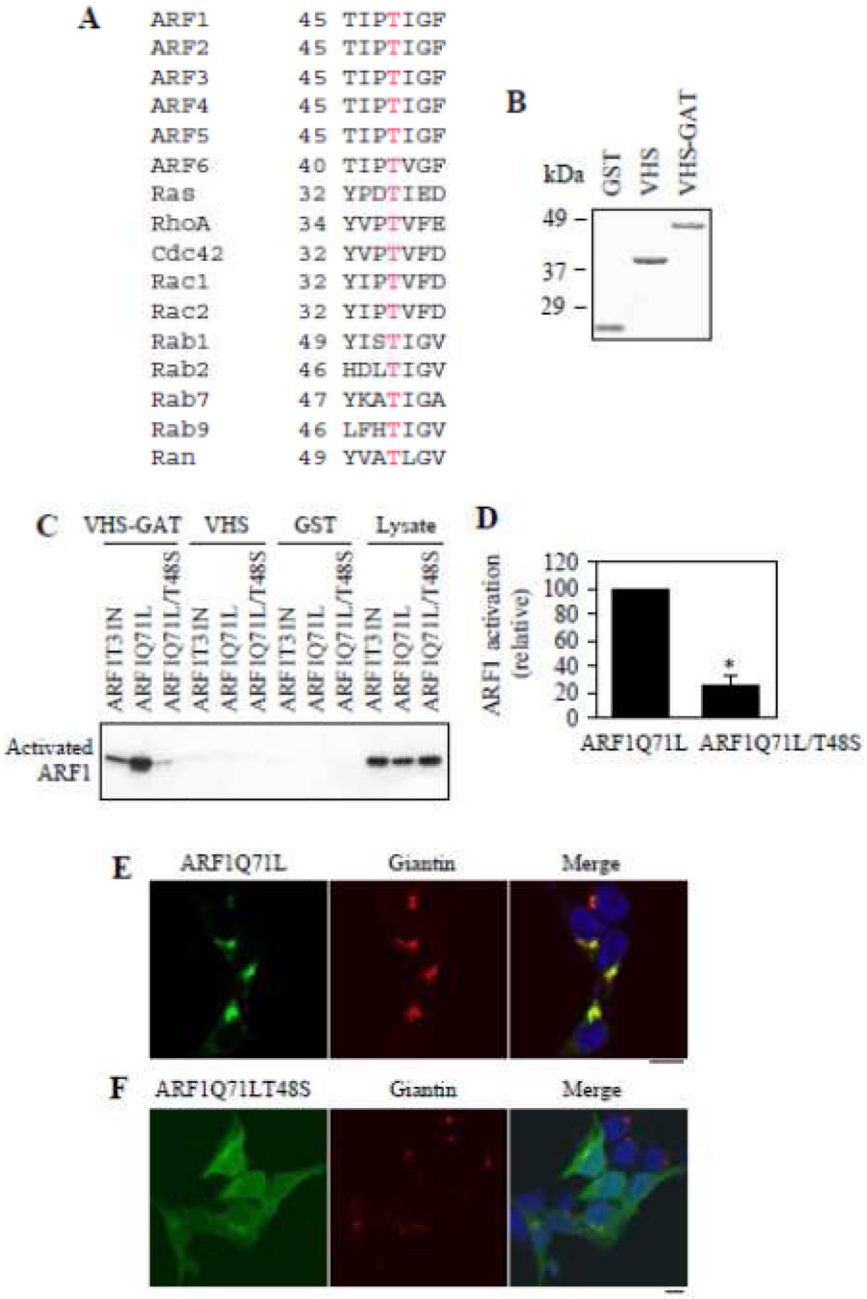

Fig. 2.

Effect of mutating Thr48 on the activation and subcellular localization of ARF1. A, An alignment of switch I regions of several Ras-like small GTPases. Thr residues are colored. B, Coomassie Brilliant Blue R250 staining of purified GST and GST fusion proteins containing the VHS and VHS-GAT domains of GGA3. Molecular mass is indicated at the left. C, Interaction of GDP-bound mutant ARF1T31N, GTP-bound mutant ARF1Q71L and double mutant ARF1Q71L/T48S with the VHS-GAT domain by GST fusion protein pulldown assays. The ARF1 mutants tagged with GFP were expressed in HEK293 cells and the cell extract was then incubated with GST fusion proteins as described under “Experimental procedures”. Bound ARF1 was determined by immunoblotting using GFP

antibodies. D, Quantitative data showing the effect of mutating Thr48 on ARF1Q71L interaction with the VHS-GAT domain. The data are expressed as percentages of ARF1Q71L bound to the VHS-GAT domain and presented as mean ± S.E. of 3 experiments. *, $p < 0.05$ versus ARF1Q71L. E and F, subcellular distribution of ARF1Q71L and ARF1Q71L/GT48S and their colocalization with the Golgi marker giantin. HEK293 cells cultured on coverslips were transfected with GFP-tagged ARF1Q71L (E) or ARF1Q71L/T48S (F). The cells were then stained with giantin antibodies (1:500 dilution) followed by Alexa594-conjugated secondary antibodies (1:300 dilution). Colocalization of ARF1 and giantin was revealed by fluorescence microscopy. Green, GFP-ARF1; red, the Golgi marker giantin; yellow, colocalization of ARF1 and giantin; blue, DNA staining by 4,6-diamidino-2-phenylindole (DAPI, nuclei). The data shown in E and F are representative images of at least five independent experiments. Scale bars, 10 μm.

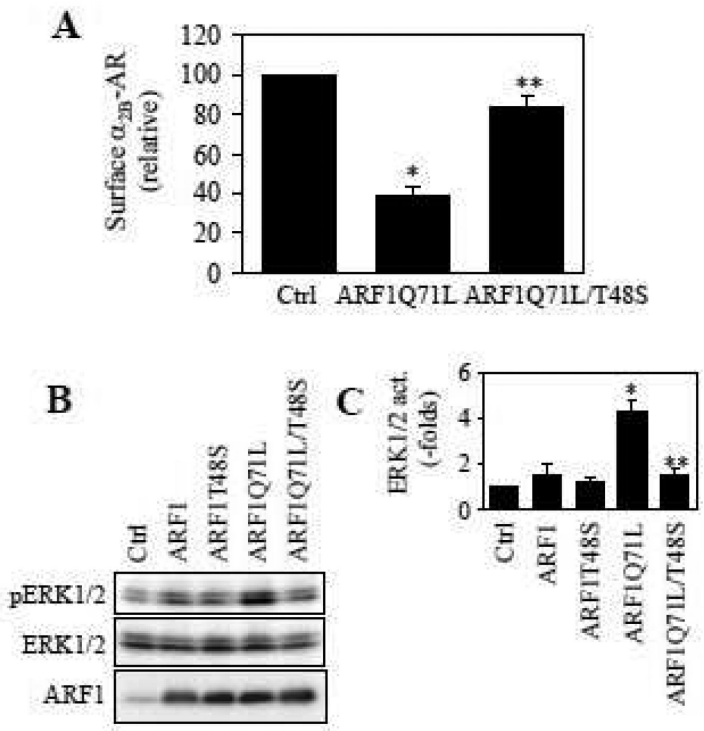

Fig. 3.

Effect of mutating Thr48 on the function of ARF1 in regulating α_{2B}-AR cell surface transport and ERK1/2 activation. A, Effect of mutating Thr48 on the inhibition of α_{2B}-AR cell surface expression by ARF1Q71L. HEK293 cells stably expressing HA-α_{2B}-AR were transfected with control vector or individual ARF1 mutant. The cell surface expression of α_{2B}-AR was determined by intact cell ligand binding using [^{3}H]-RX821002 at a concentration of 20 μM as described under "Experimental Procedures." The non-specific binding was determined in the presence of rauwolscine (10 μM). The mean values of specific [^{3}H]-RX821002 binding were 13254 ± 423 cpm from control cells. The data shown are percentages of the mean value obtained from control cells and are presented as the mean ± S.E. of three different experiments each in triplicate. * and **, $p < 0.05$ *versus* control and Q71L, respectively. B, Effect of mutating Thr48 on ARF1-mediated activation of ERK1/2. HEK293 cells were transfected with control vector, ARF1 or individual ARF1 mutants. The activation of ERK1/2 was measured by Western blotting measuring their phosphorylation using phosphor-specific antibodies as described under "Experimental Procedures". The bottom panel shows ARF1 expression by immunoblotting using ARF1 antibodies. C, Quantitative data shown in C. The data are expressed as – folds increase over the basal level and presented as mean ± S.E. of three experiments. * and **, $p < 0.05$ versue control and ARF1Q71L, respectively.

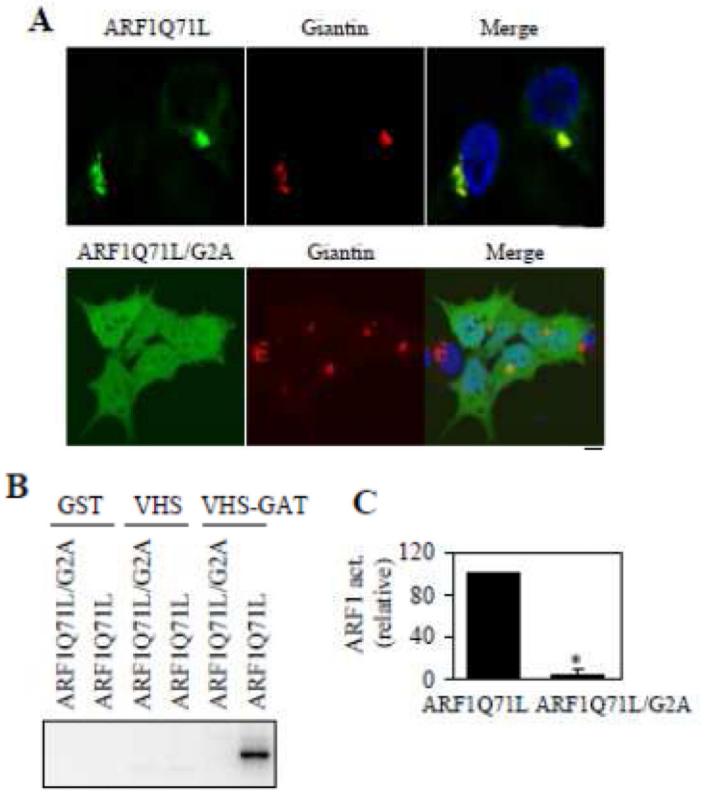

Fig. 4.

Effect of mutating Gly2 on the subcellular localization and activation of ARF1. A, Colocalization of the mutant ARF1Q71L/G2A with the Golgi marker giantin revealed by confocal microscopy as described in the legend of Fig. 2. The data shown are representative images of at least five independent experiments. Scale bar, 10 μm. B, Effect of mutating Gly2 on the activation of ARF1 measured by GST-GAT domain fusion protein pull down assays as described in the legend of Fig. 2. C, Quantitative data shown in B. The data are expressed as percentages of ARF1Q71L bound to the VHS-GAT domain. *, $p < 0.05$ versus ARF1Q71L.

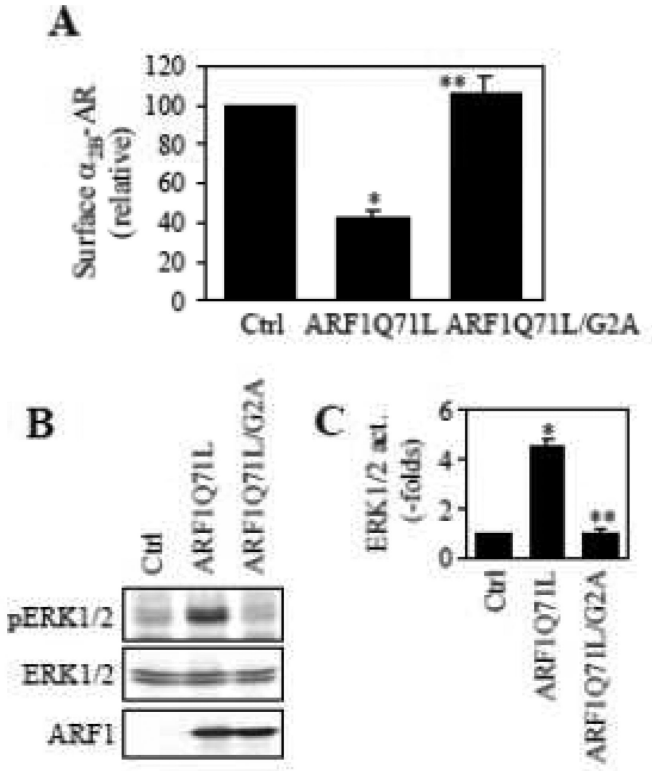

Fig. 5.

Effect of mutating Gly2 on the function of ARF1 in α_{2B}-AR cell surface transport and ERK1/2 activation. A, Effect of transient expression of ARF1Q71L and ARF1Q71L/G2A on the cell surface expression of α_{2B}-AR measured by intact cell ligand binding as described in the legend of Fig. 3. * and **, $p < 0.05$ versus control and ARF1Q71L, respectively. B, Effect of mutating Gly2 on ARF1Q71L-mediated activation of ERK1/2. HEK293 cells were transfected with control vector or ARF1 mutant. The activation of ERK1/2 was measured by Western blotting measuring their phosphorylation using phosphor-specific antibodies. The bottom panel shows the expression of ARF1 by immunoblotting using ARF1 antibodies. C, Quantitative data shown in B. The data shown in A and C are presented as means ± S.E. of at least three individual experiments. * and **, $p < 0.05$ versus control and ARF1Q71L, respectively.

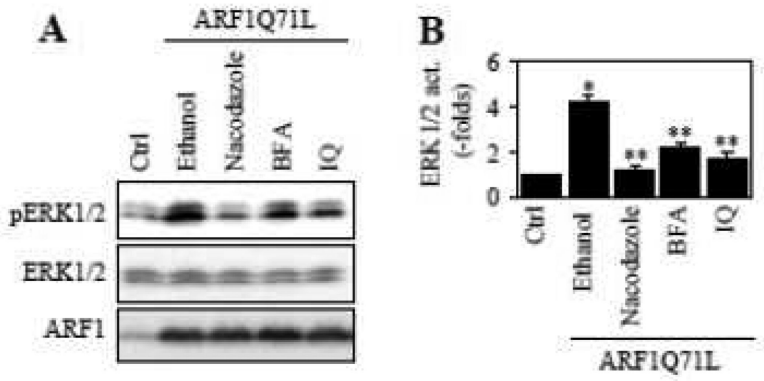

Fig 6.

Inhibition of ARF1-mediated ERK1/2 activation by Golgi structure disruptors. A, HEK293 cells transfected with control vector or ARF1Q71L. The ARF1Q71L-transfected cells were treated overnight with ethanol (control), nacodazole (1 mM), brefeldin A (BFA, 5 ng/ml), ilimaquinone (IQ, 30 μM). The activation of ERK1/2 was measured by Western blotting measuring their phosphorylation using phosphor-specific antibodies. The bottom panel shows the expression of ARF1 by immunoblotting using ARF1 antibodies. B, Quantitative data shown in A. The data are presented as means ± S.E. of at least three individual experiments. * and **, $p < 0.05$ versus control and ARF1Q71L plus ethanol treatment , respectively.

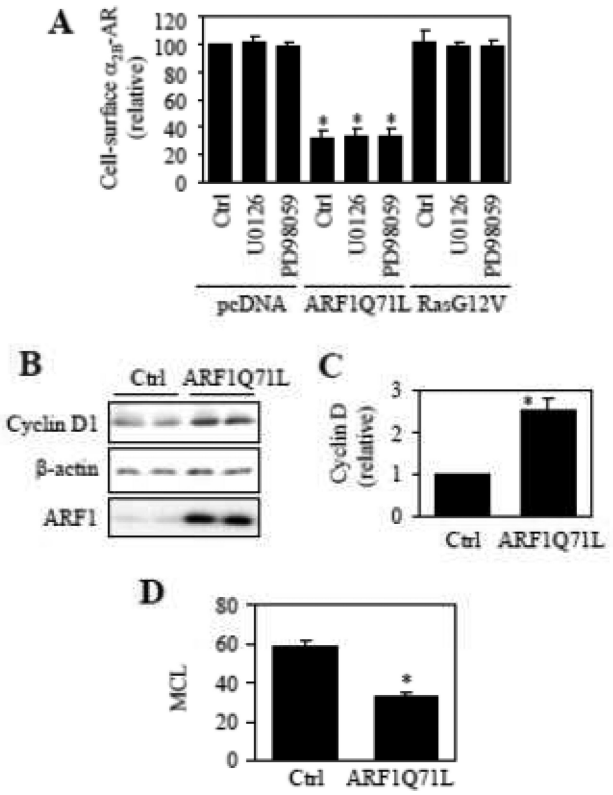

Fig. 7.

Effect of ARF1 on α_{2B}-AR cell surface transport and cell proliferation. A, Effect of ARF1 and the MAPK pathway inhibitors on the cell surface transport of α_{2B}-AR. HEK239 cells stably expressing α_{2B}-AR were transfected with control vector, ARF1Q71L or RasV12V and then treated with U0126 or PD98059 as described in the legend of Fig. 1. The cell surface expression of α_{2B}-AR was measured by intact cell ligand binding as described in the legend of Fig. 3. *, $p < 0.05$ versus cells transfected with control vector. B, Enhanced expression of cyclin D1 by expression of ARF1Q71L. HEK293 cells were transfected with control vector or ARF1Q71L and the expression of cyclin D1 was determined by immunoblotting. C, Quantitative data shown in B. The data are presented as means ± S.E. of three individual experiments. *, $p < 0.05$ versus control. D, ARF1Q71L enhances cell proliferation measured by CFSE staining as described under “Experimental procedures”. HEK293 cells were transfected with control vector or ARF1Q71L. The data are presented as means ± S.E. of three individual experiments. MCL, mean channel fluorescence. * and **, p < 0.05 versus control and ARF1Q71L, respectively.

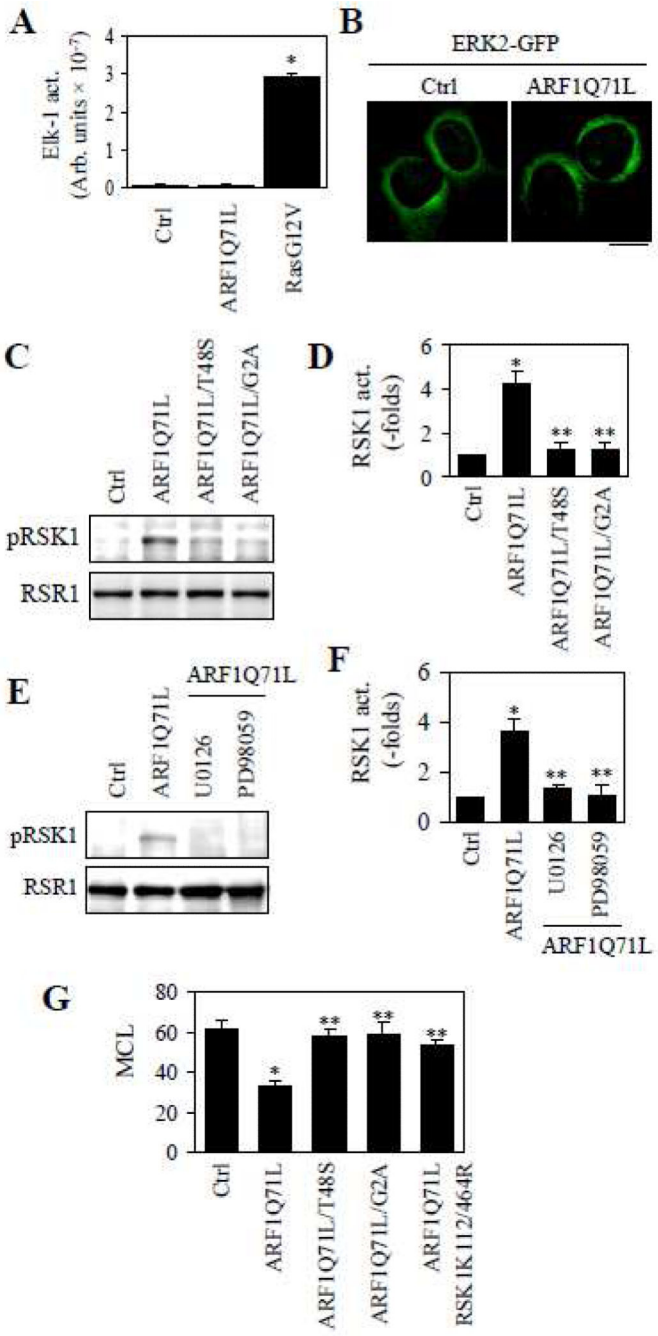

Fig. 8.

Effect of ARF1 on the activation of Elk-1 and RSK1 and subcellular localization of ERK2. A, ARF1Q71L does not activate the transcription factor Elk-1 by using the Elk-1-driven luciferase reporter system as described under “Experimental procedures”. HEK293 cells were transfected with GAL4-Elk-1 and pFR-luc together with ARF1Q71L or RasG12V for 24 h. The data are presented as means ± S.E. of ten separate experiments. B, Subcellular localization of GFP-tagged ERK2 in cells transfected with control vector or ARF1Q71L revealed by fluorescence microscopy. The data shown are representative images of at least

six separate experiments. Scale bar = 10 μm. C, ARF1Q71L activates RSK1. HEK293 cells were transfected with control vector or individual ARF1 mutant. The activation of RSK1 was measured by Western blotting measuring its phosphorylation using phosphor-specific antibodies. D, Quantitative data shown in C. The data are presented as means ± S.E. of at least three individual experiments. E, Inhibition of ARF-mediated RSK1 activation by the MAPK pathway inhibitors U0126 (10 μM) and PD98059 (50 μM). F, Quantitative data shown in E. The data are presented as means ± S.E. of three individual experiments. G, Expression of the dominant negative RSK1 mutant inhibits ARF1-mediated cell proliferation measured by CFSE staining. HEK293 cells were transfected with control vector or individual ARF1 mutant with or without cotransfection with RSK1K112/464R. The data are presented as means ± S.E. of four individual experiments. MCL, mean channel fluorescence. * and **, $p < 0.05$ versus control and ARF1Q71L, respectively.

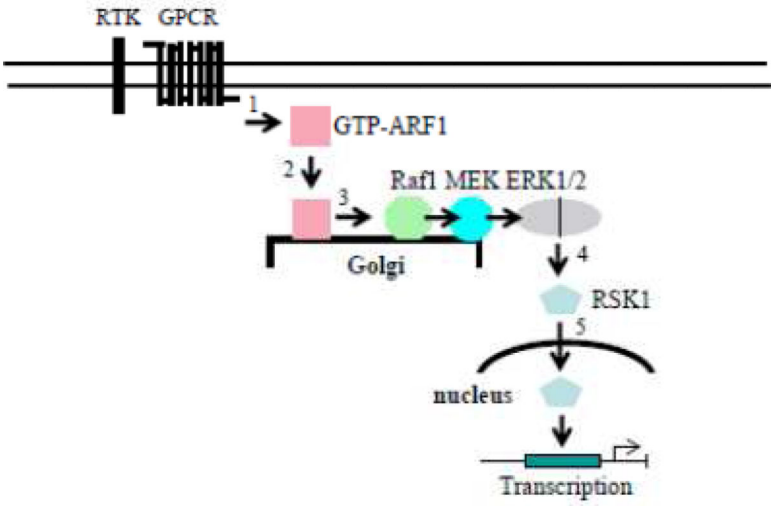

Fig. 9.

A model for ARF1 modulation of the Raf1-MEK1-ERK1/2-RSK1 pathway. 1, ARF1 activation by the cell surface receptor tyrosine kinases (RTK) or G protein-coupled receptors (GPCR); 2, ARF1 translocation to the Golgi apparatus by unknown mechanisms; 3, ARF1 activates the Raf1-MEKERK1/2 pathway likely on the Golgi compartment; 4, ERK1/2 activates the cytosolic target RSK1; 5, the activated RSK1 undergoes nuclear translocation and then induces cell proliferation.